University of Alberta

Endothelial focal adhesion remodelling following sustained lymphocyte contact

by

Sarah Catherine Mullaly

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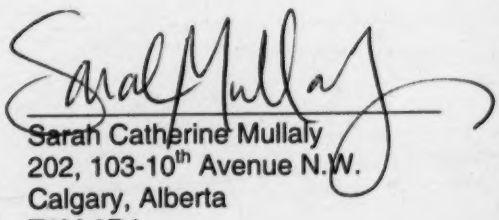

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"People know what they do; they frequently know why they do what they do; but what they don't know is what what they do does."

– Michel Foucault

University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Endothelial focal adhesion remodelling following sustained lymphocyte contact" submitted by Sarah Catherine Mullaly in partial fulfillment of the requirements for the degree of Master of Science in Experimental Medicine.

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ABSTRACT

Endothelial Focal Adhesion Remodelling Following Sustained Lymphocyte Contact

Endothelial cells (EC) actively participate in lymphocyte transendothelial migration by remodelling their actin cytoskeleton. I studied the endothelial cell matrix receptor (focal adhesion; FA) complexes to determine if these structures were remodelled following lymphocyte adhesion. Peripheral blood lymphocytes (PBL) were isolated from whole blood and added to cultured EC. Lectin-stimulated PBL adhered tightly to endothelial cells, whereas firm adhesion of freshly isolated lymphocytes to EC was induced by pre-treatment with MCP-1 or activating anti-CD11a mAb. Sustained adhesion between lymphocytes and EC resulted in a significant, contact-dependent decrease in paxillin incorporation into the focal adhesions following 15, but not 5, minutes of contact. Further, these data demonstrate that remodelling of endothelial focal adhesion structures is independent of TcR-mediated activation. Finally, I show that EC FA remodelling is independent of lymphocyte CD29, and does not occur following fibroblast adhesion, evidence that the induction of EC FA remodelling involves cellular specificity.

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LIST OF SYMBOLS AND ABBREVIATIONS

a.a.	amino acid
A	ampoule
Ab	antibody
AJ	adherens junction
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CLA	cutaneous lymphocyte-associated antigen
CSK	cytoskeleton buffer
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
ddH_20	double-distilled deionized water
DMSO	dimethyl sulfoxide
D-PBS	Dulbecco's phosphate buffered saline
D-PBS/$CaCl_2$	Dulbecco's phosphate buffered saline with 1 mM $CaCl_2$
DTH	delayed-type hypersensitivity
E#	embryonic day
EC	endothelial cell
ECGS	endothelial cell growth supplement
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELC	Epstein Barr virus-induced receptor ligand chemokine
f-actin	filamentous actin

FA	focal adhesion
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
g	gram
GALT	gut-associated lymphoid tissue
GAP	GTPase activating protein
GEF	GTP/GDP exchange factor
GDI	GDP dissociation inhibitor
GDP	guanine diphosphate
GTP	guanine triphosphate
Gln	glutamine
GlyCAM-1	glycosylation-dependent cell adhesion molecule 1
HBSS	Hanks' buffered salt solution
HEV	high endothelial venule
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
ICAM	intracellular adhesion molecule
Ig	immunoglobulin
IL-4	interleukin 4
JAM	junction-associated membrane protein/junctional adhesion molecule
kDa	kiloDaltons

L	litre
LAD	leukocyte adhesion deficiency
LIM	Lin-11, Isl-1 and Mec-3 genes
LFA-1	leukocyte function associated antigen 1
LPAM-1	lymphocyte Peyer's patch adhesion molecule 1
LN	lymph node
M	moles/litre
mAb	monoclonal antibody
MAdCAM-1	mucosal-associated cell adhesion molecule 1
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
MLSB	modified Laemmli sample buffer
P/S	penicillin/streptomycin
pAb	polyclonal antibody
PAK	p21 activated kinase
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cells
PCV	post-capillary venule
PE	phycoerythrin
PFA	paraformaldehyde
PHA-L	phytohemagglutinin
PMSF	phenylmethylsulfonyl fluoride
PNAd	peripheral node addressin

PSGL-1	P-selectin glycoprotein ligand 1
PTP	protein tyrosine phosphatase
RANTES	regulated on activation, normal, T-cell expressed, and presumably secreted
RCA	relative cell area
RFI	relative fluorescence intensity
SDF-1	stromal cell-derived factor 1
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SFK	Src-family kinase
SLC	secondary lymphoid tissue chemokine
TBS-T	Tris buffered saline-Tween20
TcR	T cell receptor
TNF	tumour necrosis factor
TJ	tight junction
V	volt
VASP	vasodilator-stimulated phosphoprotein
VCAM-1	vascular cell adhesion molecule 1
VE-cadherin	vascular endothelial cadherin
VLA-4	very late antigen 4
ZO	zonula occludens
°C	degrees Celcius

(Standard prefixes are: n, nano; μ, micro; m, milli)

CHAPTER ONE:

Background

1.1 Lymphocyte trafficking

Lymphocyte trafficking from the blood into tissues occurs during normal immune surveillance and inflammatory responses [1;2]. Peripheral blood lymphocytes (PBL) continually recirculate from the blood, through tissue, into lymph and back to the blood compartment. Recruitment from the blood is initiated by the tethering of the PBL to the vascular endothelium. Subsequently, upon receipt of a chemokine signal, PBL integrins mediate firm adhesion to the endothelium via their endothelial immunoglobulin superfamily ligands. The molecules which participate in the final transendothelial migration step of this adhesion cascade are poorly defined [3]. Interactions between the PBL and endothelium, and the specific adhesion molecules involved in these events are summarised in Table 1.1, Table 1.2 and Figure 1.1.

1.2 Lymphocyte adhesion

1.2.1 Selectins

Initial transient contact during transendothelial migration primarily involves interactions between PBL selectins and vascular endothelial addressins [4;5]. The selectins make up a family of carbohydrate-binding glycoproteins, named for their cell-specific surface expression [6;7]. Selectins have both epidermal growth

factor-type and N-terminal C-type lectin domains with multiple consensus repeat sequences [8]. Their ligands are sulfated, fucosylated mucins containing clusters of sialic acid-rich O-linked carbohydrate side chains [9]. Selectins do not require an activation signal prior to the induction of adhesion [10]. Furthermore, L-selectin is expressed on the tips of PBL microvilli, rather than on the cell body [11], which allows the PBL to make contact with the endothelium without being in tight apposition.

In addition to allowing the PBL to tether to the surface of the endothelium under shear conditions, selectin:addressin interactions also play a role in the trafficking specificity of various PBL subpopulations. Homing of naïve cells involves PBL selectins [12], whereas antigen-induced leukocyte trafficking involves endothelial selectins [13;14].

Lymphocyte homing to secondary lymphoid tissues involves interactions between L-selectin (or, CD62L) and glycosaminoglycans on its ligands, glycosylation-dependent cell adhesion molecule 1 [GlyCAM-1], mucosal-associated cell adhesion molecule 1 [MadCAM-1], podocalyxin and peripheral node addressin [PNAd; CD34] [15]. CD62L is expressed by naïve lymphocytes, and its ligands by the high endothelial venules (HEV) of lymph nodes (LN), allowing the PBL to home to sites where they can encounter antigen and become activated. Following PBL activation, CD62L is cleaved by metalloproteinase enzymes [16], preventing effector cells from entering secondary lymphoid tissues. The

exception is the "central memory" lymphocyte population which continues to express CD62L and home through secondary lymphoid tissues [17].

Inflamed endothelium upregulates surface expression of CD62E (E-selectin), for example, in cutaneous post-capillary venules (PCV) during delayed-type hypersensitivity (DTH) responses [18]. The ligand for E-selectin is CLA (cutaneous lymphocyte-associated antigen), expressed by PBL during the naïve to memory transition [19]. The third selectin, CD62P (P-selectin), is stored in intracellular granules in endothelial cells (and platelets) and is mobilised to the cell surface following agonist-induced cell activation [8]. CD62P interacts with specific carbohydrate motifs on the surface of P-selectin glycoprotein ligand 1 (PSGL-1) [15].

The functional importance of this group of molecules can be gleaned from selectin-null mice. In the absence of CD62L, peripheral lymph node size and cellularity are markedly attenuated, due to the inability of naïve lymphocytes to roll on the surface of, and thus transmigrate across, LN PCV [6;7]. Activated platelets are able to partially reconstitute PBL homing in the absence of CD62L through their expression of CD62P [20]. Platelet CD62P bridges $CD62L^{-/-}$ PBL and lymph node endothelium, associating PBL PSGL-1 and EC PNAd. Loss of functional endothelial selectins results in similar effects on PBL trafficking. Treatment of mice with anti-CD62E and CD62P Ab ablates trafficking of a specific subset of CD4+ PBL to inflamed peritoneum [14]. These effects are

likely due to the selective expression of functional CD62E/CD62P ligands by effector/memory, but not naïve, PBL [14;21]. Studies of the selectins' involvement in PBL rolling have been fundamental to the delineation of the PBL:endothelium adhesion cascade.

1.2.2 Chemokines

Chemokines are chemoattractant cytokines. Four distinct families of chemokines (C, CC, CXC, CX_3C) have been identified, into which greater than 40 chemokines are classified based on their N-terminal cysteine residues [22-24]. Chemokines interact with G-protein coupled receptors which contain seven transmembrane-spanning loops. The interaction between chemokine and receptor leads to G-protein activation via an intracellular exchange of GDP for GTP, resulting in the activation of the $G\alpha$, $G\beta$ and $G\gamma$ protein subunits. The activated G-proteins signal to downstream effectors within the cell, including various tyrosine kinases, second messengers and members of the Rho family of small GTPases [25]. The activation of various signalling cascades can result in cell shape changes [26], actin polymerisation [27] as well as the activation of integrin adhesivity [28;29].

Chemokines can be grouped into homeostatic (constitutive) and inflammatory (inducible) subsets [30;31]. Constitutive chemokines play roles in both tissue-specific PBL homing and the microenvironmental segregation of PBL subsets within tissues. Homeostatic chemokines include: stromal cell-derived factor 1 (SDF-1; CXCL12), as well as secondary lymphoid tissue chemokine (SLC;

CCL21) and Epstein Barr virus-induced receptor ligand chemokine (ELC; CCL19), amongst others. Recent work suggests that the homeostatic chemokines are largely non-redundant. The lack of SDF-1 or its receptor CXCR4 (CXC chemokine receptor 4) are perinatal-lethal mutations [32;33]. Furthermore, CCR7- (CC chemokine receptor 7; the receptor for SLC and ELC) null mice have decreased numbers of naïve T lymphocytes in secondary lymphoid compartments as well as structural defects in these sites [34]. Not unexpectedly, naïve T cells from *plt/plt* mice, which express neither SLC nor ELC [35], also display defective homing to secondary lymphoid tissues [36].

Unlike the homeostatic chemokines, inflammatory chemokines (including MCP-1 [monocyte chemoattractant protein 1; CCL2] and MIP-1α [macrophage inflammatory protein 1 alpha; CCL3]) function redundantly. As such, the phenotypes of inflammatory chemokine- or chemokine receptor-null mice are much less drastic than their homeostatic counterparts. For example, chemokine receptor CCR5- (which binds RANTES, MIP-1α, MCP-2 and MCP-3) null mice develop normally but have increased RANTES production and defective macrophage function [37;38]. Spontaneous CCR5-null mutations have also been reported in humans. Similar to the CCR5-null mouse, the CCR5-null human does not develop any obvious immunodeficiency [38].

Chemokines direct specific PBL subpopulations to discrete sites, such as the skin or the gut. Cutaneous lymphocyte-associated antigen (CLA)-positive PBL traffic preferentially to the skin, and their localisation to this site is assisted by

their expression of specific chemokine receptors. CCR4 [39] and CCR10 [40] bind cutaneous endothelium-expressed chemokines thymus- and activation-regulated chemokine (CCL17) [39] and cutaneous T cell-attracting chemokine (CCL27) [41], respectively. In contrast, gut-trafficking PBL, which express the $\alpha 4\beta 7$ integrin but not CLA, express CCR9 which binds thymus-expressed chemokine (CCL25) in the lamina propria of the small intestine [42;43]. To induce the specific localisation of PBL subsets to tissue, these chemokines trigger integrin activation to facilitate the next stage of the emigration paradigm, firm adhesion.

1.2.3 Lymphocyte integrins

In the model of transendothelial migration, integrins are responsible for inciting firm adhesion of the PBL to the endothelium [44-46]. The structures of the integrin α and β chains are outlined in Figure 1.2. Integrins are large, heterodimeric, membrane-spanning proteins consisting of noncovalently associated alpha (~1,000 a.a.) and beta (~800 a.a.) subunits, which predominantly exist in a low affinity, or avidity, state [47;48]. Each subunit contains a large extracellular domain with a globular head (~70 Angstroms in diameter) and a stalk region (~100 Angstroms), a transmembrane segment and a short cytoplasmic tail [49]. Intracellularly, integrins contain a pair of transmembrane-spanning helices with C-terminal, intracellular tails.

The regulation of integrin adhesivity states is controlled by associations of specific molecules with the C-terminal tails, resulting in inside-out and/or outside-in signalling [49;50]. Inside-out signalling arises from the intracellular compartment, and results in a change external to the plasma membrane, such as a conformational change in the integrin in response to an activation signal [51]. Outside-in signalling results in intracellular responses mediated via an external signal, for example, cytoskeletal reorganisation following integrin:ligand interaction.

Divalent cations are required for functional integrin:ligand binding [47;52]. Ca^{2+} interacts with putative binding motifs in α chains. Further, the alpha chains contain a metal ion-dependent adhesion site motif [49] and a beta-propeller structure, formed from 7 N-terminal consensus repeat sequences [47], which are also involved in integrin:ligand interactions. Some alpha chains, including αL and αM, contain a further structure involved in ligand binding known as the "I", or "inserted" domain [53]. Together these structures regulate affinity changes and integrin activation.

The major integrins involved in PBL trafficking are $\alpha L\beta 2$ (CD11a/CD18, leukocyte function associated antigen 1; LFA-1), $\alpha 4\beta 1$ (CD49d/CD29, very late antigen 4; VLA-4) and $\alpha 4\beta 7$ (CD49d/CD? [undesignated], lymphocyte Peyer's patch adhesion molecule 1; LPAM-1) [44;46;54]. LFA-1, ubiquitously expressed by

lymphocytes, associates with endothelial intracellular adhesion molecules, ICAM-1 (CD54) and ICAM-2 (CD102) [55]. ICAM-1 is expressed at low levels by resting endothelium and is upregulated upon cytokine stimulation, whereas ICAM-2 is constitutively expressed. VLA-4 is induced following PBL stimulation and associates with endothelial vascular cell adhesion molecule 1 (VCAM-1; CD106) [44]. Endothelial VCAM-1 is expressed following cytokine stimulation, and supports both rolling and arresting phenotypes [54;56]. Finally, the $\alpha 4\beta 7$ integrin, expressed by a subset of predominantly gut-trafficking PBL [57], interacts with VCAM-1 and MAdCAM-1 [54;58;59].

An activation signal is required for integrin-induced PBL adhesion [10;60;61]. Integrin activation can be induced by CD62L cross-linking, lymphocyte antigen receptor ligation, divalent cations, or most commonly, chemokine $G_{\alpha i}$-protein coupled receptor signalling [60;62]. PBL arrest, but not rolling can be inhibited *in vivo* with pertussis toxin, which ADP-ribosylates and inhibits $G\alpha i$ activity [10]. Some of the chemokines believed to be involved in PBL integrin activation are SDF-1 (CXCL12) and MCP-1 (CCL2) [44;62].

Integrin activation is believed to occur through affinity and/or avidity changes [45;53;63]. Integrin affinity changes can be induced both extra- and intracellularly. Extracellular activation can be mediated by divalent cations, such as Mn^{2+} or Mg^{2+} which have been shown to increase the binding affinities of LFA-1 and VLA-4 for soluble ICAM-1 [61] and VCAM-1 [64], respectively. Affinity

changes can also be induced *in vitro* with activating anti-integrin mAb such as TS2/16 which targets CD29 (β1 integrin) [65], and MEM-83 which targets CD11a (αL integrin) [66]. Sequences within the C-terminal domains of integrins have also been shown to play roles in integrin affinity modulation. For example, mutation of the highly conserved GFFKR sequence in the α integrin chain can result in a constitutively active affinity state [67]. Similar effects can be observed with mutations targeting β chain sequences [68]. Changes in affinity state in this manner are thought to involve conformational changes within the integrin. Upon stimulation, structures (such as the I-domain) within the alpha chain of the integrin are shifted [51], resulting in an electrophilic change. The result is an opening of the stalk region, exposing a Mg^{2+} ion which can interact with the negative charge on the surface of ICAM [69;70].

Integrin adhesivity can also be modulated by changes in avidity or macroclustering, in addition to, or in lieu of, a change in integrin:ligand binding affinity [52]. Phorbol esters, including phorbol 12-myristate 13-acetate, are able to activate LFA-1 and α5β1 integrin adhesivity through the induction of clustering and cell flattening [61]. Furthermore, induction of T cell activation through TcR cross-linking can result in changes in integrin avidity [71].

The absence of functional lymphocyte integrins can result in markedly attenuated trafficking profiles. For example, PBL adhesion to venular endothelium is

abrogated in LFA-1-null and anti-LFA-1 blocking Ab-treated mice although rolling velocity is unaffected [55;60]. The resulting phenotype in both cases is a marked blood leukocytosis and tissue leukopenia. Furthermore, the absence of LFA-1 results in defects in lymphoid tissue composition. The size of peripheral lymph nodes in the LFA-$1^{-/-}$ mouse is markedly diminished, whereas the spleen is inversely affected (due to lymphocyte redistribution) [55]. These mice exhibit normal delayed-type hypersensitivity (DTH) responses, although peripheral immune responses to cutaneous antigen are significantly inhibited (due to the inability of naïve lymphocyte to home to peripheral LN) [60;72].

Integrins can also be involved in the targeting of PBL subpopulations to specific microenvironments. The $\alpha4\beta7$ integrin is predominantly involved in PBL trafficking to the gut associated lymphoid tissue (GALT) [54], although $\alpha4\beta7^{+}$ PBL can still traffic to non-mucosal sites [57]. Unlike other α chains, $\alpha4$ integrin can initiate tethering *in vitro* in the absence of selectin:addressin interactions [54]. This ability is conferred, in part, by the surface topography of $\alpha4\beta7$ which, like CD62L, is found on the tips of PBL microvilli [54], allowing the integrin to interact with endothelial ligands in the absence of tight contact.

The absence of the $\beta7$ integrin, which can interact with both $\alpha4$ and αE, results in defective GALT formation (Peyer's patch hypoplasia) [73]. The number of intra-epithelial lymphocytes surrounding the epithelial cells in the GALT is also

decreased [73]. These defects are likely due to defective lymphocyte trafficking, as lymphocyte development is unaffected [73-75].

It is important to note that these integrin-null phenotypes are not attributable to ineffective PBL transendothelial migration and that the defect is, in fact, in the number of cells able to adhere to the endothelium following tethering [55]. Taken together, this confirms that PBL selectin:endothelial addressin interactions precede integrin:immunoglobulin superfamily associations, which precede emigration to the extravascular compartment.

1.2.4 Leukocyte adhesion deficiency

In humans, the lack of functional leukocyte adhesion molecules can manifest in severe immunologic disorders, including leukocyte adhesion deficiency types I and II (LAD I, LAD II) [76]. LAD I results from a mutated CD18 (β2 integrin) gene [77]. Clinical manifestations include delayed umbilical cord detachment (which can act as an as early indicator of LAD I), as well as impaired wound healing and severe neutrophilia. Patients develop recurring fungal and bacterial infections, and infection-resultant mortality usually occurs in severe forms of LAD I.

The second form of LAD (LAD II) results from a defect in the selectin pathway, specifically the enzyme responsible for converting mannose to fucose, GDP-D-mannose-4, 6 dehydratase (GMD) [78]. This results in disrupted synthesis of the Sialyl Lewis X motifs in the ligands for CD62E and CD62P [79]. The

immunologic phenotype of this disorder is less severe compared to that of LAD I or the CD62E/CD62P-null mouse, although other organ systems are affected due to the defect in fucose metabolism.

1.3 Diapedesis

In vitro, leukocyte migration terminates at interendothelial cell junctions where the final step of diapedesis occurs [80]. After the leukocyte adheres to the surface of the endothelium, it migrates laterally to an intercellular junction [81;82] where it then emigrates to the extravascular compartment. Leukocytes have been shown to extend a pseudopod rich in LFA-1 between adjacent endothelial cells as they commence diapedesis [3]. This pseudopod also contains high levels of filamentous (f-) actin, suggesting roles for both of these components in transendothelial migration. Leukocyte treatment with activating anti-integrin mAb has been shown to block the lateral migration of these cells over EC monolayers [83;84] and also decreases their ability to undergo transendothelial migration [80], confirming the involvement of integrins in diapedesis.

Platelet/endothelial cell adhesion molecule 1 (PECAM-1; CD31) was thought to play a key role in the initiation of leukocyte transmigration based on blocking studies performed both *in vivo* and *in vitro* [82;85] however, the phenotype of the null mouse showed no defects in leukocyte adhesion or extravasation [86].

Leukocytes have also been observed to diapedese transcellularly in electron microscopy studies [87;88]. The reliance of leukocytes on this transmigratory path has been questioned by other studies which have shown both leukocyte transmigration at interendothelial junctions, and displacement of interendothelial junction proteins during diapedesis [3;85;89]. Very little work has focussed on the specific signalling events within PBL during transendothelial migration.

1.4 The endothelium

In humans, it is believed that as many as $3x10^8$ lymphocytes migrate through specialised vascular beds known as high endothelial venules (HEV) into secondary lymphoid tissue per minute [90]. The HEV are the primary trafficking site in secondary lymphoid tissues, excepting the spleen where PBL emigrate through blood sinusoids within the marginal zone [90;91]. HEV EC display a cuboidal morphology, are highly metabolically active and are rich in rough endoplasmic reticulum, golgi and polyribosomes [90]. Additionally, HEV EC contain secretory vesicles, referred to as Weibel-Palade bodies. These bodies contain proteins including CD62P and von Willebrand factor, which can be mobilised to the surface of the endothelium pursuant to inflammatory stimuli [92]. Furthermore, HEV EC express specific adhesion molecules for naïve, and a subset of memory, PBL not expressed in other vascular endothelial beds that allow these PBL to traffic efficiently to these sites [91;93;94].

When peripheral lymph nodes are starved of afferent lymph, HEV EC revert to a flat morphology and no longer support high-volume PBL transmigration [95]. High endothelial morphology is restored following the injection of antigen directly into the lymph node. The enhanced production of cytokines and chemokines following the entry of antigen may also be involved in maintaining HEV morphology [90]. The presence of inflammatory stimuli can also induce the upregulation of surface adhesion molecules, including CD62E [18] and CD106 [96], by other vascular beds to assist in the trafficking of lymphoid cells, such as effector T lymphocytes, to sites of inflammation.

Endothelial cells contain multiple adhesion structures involved in formation and maintenance of vast vascular networks, anchoring the EC to each other and to their underlying matrix [97;98]. The predominant structures linking the EC laterally are the tight junctions and the adherens junctions (Figure 1.3). Tight junctions regulate paracellular permeability and are predominantly localised to sites where the endothelium must stringently control exchange between the blood and tissue compartments, for example at the blood-brain barrier, and in large arteries (i.e. in intestinal, cardiac and muscular endothelia) [99]. In contrast, tight junctions are largely absent from sites of high volume leukocyte transmigration, including post-capillary HEV. Adherens junctions are ubiquitously expressed between adjacent EC and are also involved in paracellular permeability, as well as the maintenance of structural stability within the

endothelial monolayer [100]. Finally, linking the EC to the basement membrane is the matrix receptor complex (Figure 1.4 and Section 1.5.3) [101].

These adhesion complexes are linked intracellularly to the actin framework of the cytoskeleton, which assists in the regulation of the complexes' functionality [102]. Together these structures, by maintaining structural competence and reducing vascular leakage, may also act to impede PBL transmigration.

1.5 Interendothelial junctions

1.5.1 Tight junctions

Tight junctions (TJ; zonula occludens) were initially characterised in the electron microscopy studies of Farquhar and Palade in 1963, although the existence of such a barrier had been hypothesised by Bonnet in 1895 (reviewed in [99]). Tight junctions are the most luminal of the adhesive complexes. They are found in endothelium *in vivo* [103], and can be induced *in vitro* in confluent human umbilical vein EC (HUVEC) treated with astrocyte-conditioned media [104]. At least nine TJ components have been characterised, although all are not present in endothelium.

1.5.1.1 Integral membrane components of the tight junction

The characterised integral membrane proteins in endothelial tight junctions include the claudin family (claudin-1, -2 and -5) [105], occludin [106] and members of the junction-associated membrane protein/junctional adhesion

molecule (JAM) family [81]. Claudins and occludin each contain four membrane-spanning domains [105;106]. The JAM proteins are members of the immunoglobulin superfamily and contain a single transmembrane pass [81]. Whether JAM proteins are actually constituents of the TJ or are co-localised to this site is undetermined.

1.5.1.2 Intracellular components of the tight junction

The structure of the integral membrane proteins claudin and occludin allows for both the N- and C-termini to be oriented cytoplasmically, where they can interact with the intracellular components of the TJ. The intracellular proteins connect the integral membrane components of the tight junction to the dense peripheral band, comprised of f-actin, myosin and α-actinin [102]. Certain members of the zonula occludens family associate directly with the integral membrane proteins, although they do not contain enzymatic abilities [99]. Other proteins which associate with the TJ include cingulin and 7H6 antigen.

1.5.1.3 The tight junction in leukocyte transendothelial migration

Studies surrounding the response of EC tight junctions to transmigrating leukocytes have predominantly been done *in vitro* using HUVEC monolayers induced to form TJ in the presence of astrocyte-conditioned media. In these systems, the majority of neutrophils tend to diapedese preferentially at sites of discontinuous or absent TJ [107]. Further, neutrophil transendothelial migration does not appear to result in the proteolysis of tight junction occludins, or zonula

occludens proteins when these cells are seeded on endothelium [104]. Whether leukocyte extravasation *in vivo* results in proteolytic degradation of endothelial tight junction components has yet to be completely ascertained.

The involvement of tight junction components in directing leukocyte transendothelial migration has also been addressed. Martin-Padura et al. [81] have shown that treatment of cultured EC with blocking mAb against JAM (BV11) leads to a significant inhibition of both spontaneous and chemokine-induced monocyte transendothelial migration *in vitro*. This suggests that endothelial adhesion molecules localised to intercellular junctions may play key roles in leukocyte diapedesis *in vitro* and extravasation *in vivo*. A similar result was observed in PECAM-1 blocking studies [82], subsequently questioned by the characterisation of the null mouse [86]. Whether leukocyte trafficking in the JAM-null mouse will parallel, or cast doubt upon, the results of the blocking studies has yet to be determined.

1.5.2 Adherens junctions

Adherens junctions (AJ; zonula adherens) can be found in endothelium both *in vitro* [89] and *in vivo* [100]. AJ are abluminal to the tight junctions and are composed of integral membrane cadherins and multiple cytosolic proteins which anchor the complex to the actin cytoskeleton.

1.5.2.1 Integral membrane components of the adherens junction

Cadherins, the sole integral transmembrane constituent of the adherens junction, are calcium-dependent, single-chain adhesion molecules [108]. The cadherins contain an extracellular domain consisting of five repeating domains, a single transmembrane-spanning segment, and a highly conserved intracellular domain which associates with the actin cytoskeleton through other AJ proteins [109]. Cadherins associate both as side-by-side dimers within a single cell, and as anti-parallel structures linking adjacent cells. It has been proposed that this organisation is sequential, with the formation of dimers preceding the anti-parallel associations between neighbouring cells. This linearity is believed to be controlled by the requirement for both calcium and intracellular adherens junction proteins for the formation of lateral associations but not dimerisation [109]. Chelation of extracellular calcium causes the loss of cadherins (and catenins) from the adherens junctions, although vascular integrity remains intact under non-shear conditions. Following the application of shear stress (6 dyn/cm^2, 60 min), however, intercellular junction integrity is attenuated [109].

Cadherins are classified by the tissue in which they are expressed and include the vascular endothelial (VE), placental (P), neuronal (N) and epithelial (E) subtypes amongst others [102]. EC express VE-cadherin, which is organised into adherens junctions and shows homology with other cadherins, with minor modifications in the cytoplasmic and extracellular domains [100]. Endothelium also expresses N-cadherin, although it is not organised into adherens junctions

but is found diffusely throughout the cytoplasm [110]. Additionally, brain endothelium expresses E-cadherin [98]. VE-cadherin is the primary mediator of endothelial adherens junction signalling, and in its absence, endothelial precursor cell organisation into vascular structures is ablated [108]. Furthermore, inhibition of VE-cadherin through intravenous injection of mAb BV13 results in an increase in vascular permeability, edema and leukocyte infiltration into the heart and lungs [100].

1.5.2.2 Intracellular components of the adherens junction

Cadherins are associated intracellularly with the dense peripheral band via interactions with catenins (α, β, γ and p120 catenin [$p120^{ctn}$]) and α-actinin [109]. β-catenin, γ-catenin (plakoglobin) and $p120^{ctn}$, but not α-catenin, associate directly with the cadherins. β- and γ-catenin associate with a distal portion of the cadherin's intracellular domain, whereas $p120^{ctn}$ associates with the intracellular juxtamembrane segment. α-catenin (a vinculin homologue) associates directly with β- and γ-catenins, as well as α-actinin, but not $p120^{ctn}$ or the cadherin (Figure 1.3) [100;109]. Other proteins which associate with the AJ, distal to the cadherins, include vinculin, zonula occludens-1 and moesin [98].

Catenin phosphorylation is believed to regulate the assembly and disassembly of adherens junctions. Briefly, when β-catenin becomes tyrosine phosphorylated, cadherins are uncoupled from the actin cytoskeleton [111]. The maintenance of

catenins in an unphosphorylated state is thought to be mediated by the non-receptor protein tyrosine phosphatase, PTP1B, which is active when phosphorylated (possibly by Fer, a non-receptor protein tyrosine kinase) [111].

1.5.2.3 The adherens junction in leukocyte transendothelial migration

Adherens junctions undergo dynamic remodelling during leukocyte diapedesis. It was initially believed that neutrophil adhesion and subsequent diapedesis across cytokine-activated endothelium was facilitated by degradation of adherens junction cadherins and catenins [112]. These effects were subsequently shown to be artefactually-induced by neutrophil proteases mobilised during the lysis procedure [113].

By transfecting cultured EC with a VE-cadherin-enhanced green fluorescence protein fusion protein, leukocyte adhesion and diapedesis have been observed to result in the formation of transient gaps between adjacent EC. In contrast to the observations made in previous studies [112], VE-cadherin was not degraded, but displaced around the transmigrating leukocyte. Following leukocyte emigration, adherens junction integrity was restored and pores were sealed [89]. Monocytic THP-1 cells have also been shown to induce the transient redistribution of endothelial adherens junction components during diapedesis [3]. The exact mechanism(s) underlying leukocyte-induced adherens junction remodelling have not been elucidated.

1.5.3 The matrix receptor complex

Matrix receptor complexes, characterised in electron microscopy and interference reflection microscopy studies in the 1970s [114], are located on the luminal and basolateral surfaces of the endothelium. Focal adhesions (FA) are composed of transmembrane-spanning integrin heterodimers and associated intracellular proteins which interact with the actin stress fibres of the cytoskeleton. FA display remarkable structural heterogeneity, with multiple distinct subtypes having been characterised (elaborated in Section 1.5.3.3) [114-116]. FA are found in endothelium both *in vivo* [101;103;117] and *in vitro* [118;119], and attach the endothelium to the basement membrane. Disruption of integrin-mediated adhesion can result in anoikis (detachment-induced cell death) [120;121]. From the Greek meaning "homelessness", anoikis also prevents the formation of inappropriate endothelial islands of previously-detached EC amidst the vascular network.

1.5.3.1 Integral membrane components of the matrix receptor complex

Endothelial adhesion to the basement membrane is mediated via transmembrane, heterodimeric β1 and β3 integrins. These integrins associate with specific extracellular matrix (ECM) ligands, including type I collagen (α2β1), fibronectin (α5β1), vitronectin (αvβ3) and laminins (α6β1) [49;101;115;122], although some integrins can interact with multiple ligands [123]. Within their fibronectin or vitronectin ligands, integrins associate with the RGD (arginine-glycine-aspartic acid) sequence [124]. Subsequent clustering results in integrin

activation and FA formation, events in which integrin cytoplasmic domains are crucial. Deletion or mutation of the integrin β chain cytoplasmic domain inhibits integrin recruitment and clustering [123]. The integrin β chain appears to be fundamental to other intracellular processes as well, including focal adhesion kinase activation, integrin "cross-talk" and cell motility (also involving the α chain). In addition to a role in cell motility, the integrin α chain is thought to inhibit β chain function prior to integrin:ECM interactions [125], an effect which is subsequently ablated following ligation.

Similar to PBL integrins, endothelial integrin heterodimers participate in both inside-out signalling, which regulates attachment to the ECM, and outside-in signalling, which facilitates the development of FA [67;123;125;126]. Miyamoto et al. have shown that both ligand occupancy and clustering are involved in FA assembly. Through the use of polystyrene beads coated in cluster-inducing mAb, focal adhesion kinase and tensin were shown to associate with the integrins. When beads coated with both substrate and clustering mAb were used, there was enhanced association of cytoskeletal proteins with the integrins [127].

Associated with endothelial integrins are the integral membrane proteins syndecan-4 and the tetraspanins. Syndecan-4, or ryudocan, is a member of the syndecan family of heparan sulfate proteoglycans. It associates with the

heparin-binding domain of fibronectin and may assist in FA stabilisation. Fibroblasts derived from syndecan-4-null mice develop normal FA, indicating that this protein is not essential for FA formation [128]. Tetraspanins are members of the transmembrane 4 superfamily which interact with the extracellular domains of integrins [129] in focal contacts but not focal adhesions [130]. These molecules, which include CD9, CD63 and CD151, are believed to promote interactions between the integrins and the cytoskeleton, possibly through their effects on phosphoinositol signalling [130;131].

1.5.3.2 Intracellular components of the matrix receptor complex

Directly associated with the intracellular face of the integrin heterodimers are α-actinin and talin [125;132]. These proteins are further associated with tensin, vinculin and paxillin, which through other associations, link the integrins to the actin cytoskeleton [114;115]. Other less abundant proteins located within endothelial focal adhesions include zyxin, vasodilator-stimulated phosphoprotein (VASP), profilin and fimbrin [125;133]. Zyxin associates with VASP, which binds profilin, and together this complex plays a role in actin polymerisation during FA assembly [125]. Other signalling proteins associated with the FA include focal adhesion kinase (pp125FAK), Src-family kinases and members of the Rho family of small GTPases [134-136]. The role of tyrosine kinases and Rho family members in FA signalling is discussed in Section 1.5.3.4.

Vinculin is a highly abundant, 117 kDa FA protein which associates directly with other FA proteins, α-actinin and talin, via its N-terminus [137]. The C-terminus of vinculin associates with paxillin, acidic phospholipids (including phosphatidylinositol 4,5-bisphosphate; PIP_2) and the actin cytoskeleton. PIP_2 is required for FA associations, as it induces a conformational change in vinculin which allows it to associate with talin and actin [138]. In addition to its localisation in FA, vinculin has also been shown to associate with the intracellular components of adherens junctions [98].

Deletion of the vinculin gene is an embryonic-lethal (by day E10) mutation due to ablated neural and cardiac development [137]. The vinculin gene is a single copy with only one splice variant which, along with the strong phenotype of the null mouse, indicates the absence of another protein which could rescue the deletion. Fibroblast cells derived from the null mouse also display dysmorphic phenotypes. These cells have decreased adhesion to ECM substrata and display increased rates of migration [137]. Although FA can be observed, due to compensatory roles of other proteins, these cells tend not to develop lamellipodia, characteristic of motile or spreading cells. Further, these cells contain significantly augmented levels of focal adhesion kinase (discussed in Section 1.5.3.4) [137]. From these observations, a role for vinculin in both structural and signalling events is evident.

Paxillin, a member of the LIM family (named from the Lin-11, Isl-1 and Mec-3 genes), is a 68 kDa multidomain protein which localises to the integrin-adjacent plasma membrane within the FA [139;140]. Similar to the vinculin-deficient mouse, the paxillin-null is an embryonic-lethal (by day E7-8) [139], although the complete phenotype is uncharacterised. Paxillin targeting to, and anchoring at, the cell membrane is mediated by its C-terminal, serine- and threonine-phosphorylated double zinc-finger LIM domains (LIM1-4) [139;141]. The N-terminus of paxillin contains highly conserved leucine-rich LD motifs which act as docking sites for other cytoskeletal/signalling proteins including pp125FAK, proline-rich tyrosine kinase 2 and vinculin [139]. The N-terminus also contains putative Src-homology 3- and Src-homology 2-binding motifs which allow pp125FAK and FAK-associated Src to associate with and phosphorylate paxillin tyrosine residues 31 and 118 [142;143].

Phosphorylation of tyrosine residues in its N-terminus by Src or FAK provides paxillin-docking sites for other adaptor proteins such as Crk and p130Cas [139]. The recruitment of Crk and phosphorylated Cas results in the activation of the mitogen-activated protein kinase signal cascade, and the induction of gene transcription. The Src inhibitor, C-terminal Src kinase, and the p130Cas protein tyrosine phosphatase PEST can also associate directly with paxillin to alter phosphorylation states of various substrates, including Src and p130Cas, to terminate signalling [139;144].

In addition to stimulation of gene expression, paxillin is also involved in the regulation of FA and cytoskeletal integrity. PAK (p21 activated kinase) is associated with paxillin at the FA, and this localisation facilitates its roles in cytoskeletal remodelling [145]. PAK is a downstream effector of Rho family member, Rac1 (discussed in Section 1.5.3.4) and signals to other proteins including LIM-kinase [146] and myosin light chain kinase [147] to facilitate changes in the cytoskeleton. Taken together, this supports the notion of paxillin as both a structural and signalling/adaptor protein, the full implications of which have not yet been characterised.

1.5.3.3 Matrix receptor complex assembly, maturation and diversity

FA complexes begin to form within an hour following cell:matrix associations [116;148] although FA formation has been observed as early as 15 minutes following cell adhesion in other experimental systems [149]. These complexes form at the cell periphery as small, nascent focal contacts, and subsequently develop into mature FA following associations with the actin stress fibres of the cytoskeleton [116]. Mature focal complexes confer increased adhesivity and isometric tensile strength to the cell from within the attachment sites. The assembly and dynamic nature of FA has recently been addressed in real-time in quiescent fibroblasts [116;150]. FA in stationary, non-motile fibroblasts are dynamic, highly motile structures. Following focal complex formation, the structures migrate in an actomyosin-dependent manner into an inner culling zone concomitant with the formation of new complexes at the cell edge [116].

Conversely, in motile cells, FA at the leading edge remain fixed, conferring traction to the cell while it moves over the surface. As the cell extends over and beyond, those complexes at the trailing edge of the cell are released [150]. A "molecular clutch", reflecting the rheostat between tension and adhesion, has been proposed to account for these observations [150].

Three types of FA have been characterised based on their constituents, size and axial ratios [114]. "Fibrillar adhesions" display an elongated morphology and contain high levels of tensin, but low levels of paxillin, vinculin and phosphotyrosine. "Classical" FA are chevron-shaped and contain high levels of paxillin, vinculin, f-actin bundles and phosphotyrosine but low tensin. Formation of classical FA has been shown to be dependent upon tensile strength, conferred by both the ECM and the intracellular cytoskeleton [115]. Finally, "mosaic adhesions" physically resemble the classical FA, but are intracellularly chimeric, containing constituents of both fibrillar and classical FA.

The type of FA formed following adhesion to the substratum can be affected by specific properties of the associated ECM proteins. Katz et al. [115] have shown that by altering the physical properties of the fibronectin ECM, different types of focal complexes form. Fibrillar adhesions are observed following association between the fibroblast $\alpha 5\beta 1$ integrin and fibronectin fibrils. In contrast, in the presence of immobilised fibronectin, the $\alpha 5\beta 1$ integrin associates into classical

focal contacts (similar to αvβ3-based FA which associate with a vitronectin matrix).

1.5.3.4 Signalling in matrix receptor complex assembly and disassembly

FA are dynamic structures, and the regulation thereof involves both the activity of the Rho family of small GTPases [119;136] and tyrosine phosphorylation events mediated by Src-family kinases and focal adhesion kinase [134;143;151;152].

RhoA and Rac1 are members of the Rho family of small (20-25 kDa) GTPases, and cycle between the active (GTP-bound) and inactive (GDP-bound) states, based on interactions with upstream activators (GEF; GTP/GDP exchange factors), inactivators (GAP; GTPase activating proteins) and GDP dissociation inhibitors (GDI) [153;154]. RhoA functions as a key regulatory molecule and, when active, is involved in the formation of stress fibres and FA [136]. GTP-RhoA activates phosphatidylinositol-4-phosphate 5-kinase (PI4P-5 kinase) to generate PIP_2 [155]. PIP_2 induces conformational changes in vinculin, ezrin, radixin and moesin, exposing their actin binding sites, resulting in the formation of FA [138;155]. Active RhoA also associates with the effector mDia (a mammalian homologue of diaphanous) which activates the small protein, profilin [156;157], resulting in actin polymerisation. GTP-RhoA activates other downstream kinases, such as the serine/threonine kinase, Rho-kinase, leading to the activation of myosin light chain kinase and the inhibition of myosin light chain phosphatase [158]. Together these effects result in increased phosphorylation of

the regulatory chain of myosin II and actomyosin bundling. Further, Rho-kinase has been shown to activate LIM-kinase, which phosphorylates and inactivates cofilin, preventing actin depolymerisation [157]. The end result is an increase in stress fibre and FA formation [159] and the prevention of actin/myosin disassembly. The activation of RhoA during FA formation has been shown to be a biphasic response. That is, immediately following integrin adhesion to the ECM, RhoA is inactive (GDP-bound). RhoA subsequently becomes activated (GTP-bound), resulting in the induction of FA formation [160].

Rac1 acts antithetically to RhoA in matrix adhesion dynamics in some systems [161]. Rac1 activation results in cell spreading and the formation of lamellipodia [119], whereas RhoA activation infers cell contractility [162]. Because of their opposing roles, it is not surprising that Rac1 can also act as a negative regulator of RhoA. Sander et al. have shown that in the presence of either dominant active forms of Rac1, or through the inhibition of the Rac1GAP TIAM-1, RhoA activity is diminished [161]. In agreement with these findings, the inactivation of Rac1 results in RhoA activation [163]. Inhibition of RhoA is also observed following the activation of a third member of the Rho family, Cdc42 [119;161].

Tyrosine phosphorylation events mediated by Src-family kinases (SFK) and focal adhesion kinase play key roles in the negative-regulation of FA dynamics. SFK have been shown to disrupt FA assembly, and in the absence of pp60Src, formation of FA is enhanced [151]. Similarly, in cells null for three SFK, pp60Src,

pp59Fyn and pp62Yes (an embryonic-lethal mutation by day E9.5), FA display decreased tyrosine phosphorylation and motility, suggesting enhanced FA formation [151;164]. In agreement with these findings, v-Src results in enhanced FA disassembly through the indirect inactivation of RhoA [135]. V-Src is a constitutively activated form of c-Src which lacks a regulatory tyrosine residue near its C-terminus [165]. Upon dephosphorylation, the normal, phosphorylated c-Src becomes active. V-Src is akin to constitutively dephosphorylated c-Src and is therefore chronically active. V-Src localises to FA and its catalytic activity stimulates the binding of p120RasGAP to tyrosine phosphorylated p190RhoGAP, shown by immunoprecipitation and immunoblotting. The association results in p120-associated p190RhoGAP activity, followed by the inactivation of RhoA, quantified through a % GTP hydrolysis assay [135].

Focal adhesion kinase (pp125FAK) plays a similar role in the regulation of FA integrity. pp125FAK is a non-receptor protein tyrosine kinase located within the matrix receptor complex [166;167]. Absence of FAK is a lethal mutation; however, FAK-null fibroblasts develop large FA and display decreased motility [134]. Paradoxically, c-Src deficient cells have increased pp125FAK present at the FA [151]. FAK has been shown to be have an inhibitory effect on Rho [168], and furthermore, becomes activated immediately following integrin:receptor ligation during which RhoA is inactivated. A model is presented wherein immediately following integrin ligation to the extracellular matrix, FAK (and possibly SFK) becomes activated. Shortly thereafter, FAK is inhibited

concomitant with the activation of RhoA, resulting in the formation of large focal plaques [160;168]. This type of model would explain the discrepancy between the early activation of FAK following attachment and its inhibitory effect on FA assembly.

1.5.3.5 Lymphocytes, the endothelial matrix receptor complex and endothelial adhesion

In this laboratory, it has been shown that, following prolonged lymphocyte adhesion, EC detach from their underlying matrix [65]. The effect is cell-contact dependent, as it is abrogated when the PBL are separated from the EC by a Transwell filter. Most importantly, PBL-induced EC detachment can be inhibited by restraining the affinity-state of the EC β1 integrin, alluding to the importance of modulation of integrin activation in EC attachment and signalling.

Lymphocyte adhesion and cross-linking of endothelial surface adhesion molecules has also been shown to induce signalling within the endothelial FA complex. Etienne et al. [169] have shown that the cross-linking of endothelial CD54 induces RhoA-regulated tyrosine phosphorylation of pp125FAK, paxillin and p130Cas. Furthermore, CD54 cross-linking results in an intracellular calcium flux [170]. This calcium flux results in activation of pp60Src, tyrosine phosphorylation of actin-associated (and presumably, FA-associated) proteins, and cytoskeletal rearrangements. Most importantly, these events appear to be required for PBL diapedesis.

1.6 Specific goals

The role of the endothelium in facilitating PBL transmigration has largely been focussed on the lateral wall junctions. Very little work has addressed the link between the EC and its basement membrane, the matrix receptor complex. I hypothesized that sustained lymphocyte contact would result in altered endothelial FA integrity by a cell contact-dependent mechanism. Further, I sought to elucidate some of the PBL requirements for the induction of endothelial matrix receptor remodelling.

1.7 Tables

Table 1.1. Table of adhesion molecules and their counter-receptors involved in lymphocyte:endothelial interactions

LYMPHOCYTE	ENDOTHELIUM	FUNCTION
L-selectin (CD62L)	PNAd (CD34), MAdCAM-1, GlyCAM-1	Rolling
CLA	E-Selectin (CD62E)	Rolling
PSGL-1	P-Selectin (CD62P)	Rolling
LFA-1 (CD11a/CD18; $\alpha L\beta 2$)	ICAM-1 (CD54) ICAM-2 (CD102)	Arrest
VLA-4 (CD49d/CD29; $\alpha 4\beta 1$)	VCAM-1 (CD106)	Rolling/Arrest
LPAM-1 (CD49d/CD?; $\alpha 4\beta 7$)	MAdCAM-1	Rolling/Arrest

Adapted from [171].

Table 1.2. Surface expression of adhesion molecules involved in lymphocyte:endothelial interactions

ADHESION MOLECULE	SURFACE EXPRESSION
1. Selectins	
CD62L	All leukocytes except effector and some memory subtypes
CD62E	Endothelial cells (inducible)
CD62P	Endothelial cells, platelets (inducible; mobilised from Weibel Palade bodies)
2. Integrins	
αLβ2 (LFA-1)	All leukocytes
α4β1 (VLA-4)	Most leukocytes (inducible)
α4β7 (LPAM-1)	Lymphocytes and other leukocytes
3. Integrin ligands: Immunoglobulin Superfamily	
CD54 (ICAM-1)	Most cells (inducible)
CD102 (ICAM-2)	Endothelium, platelets (constitutive)
CD106 (VCAM-1)	Endothelial cells (inducible)

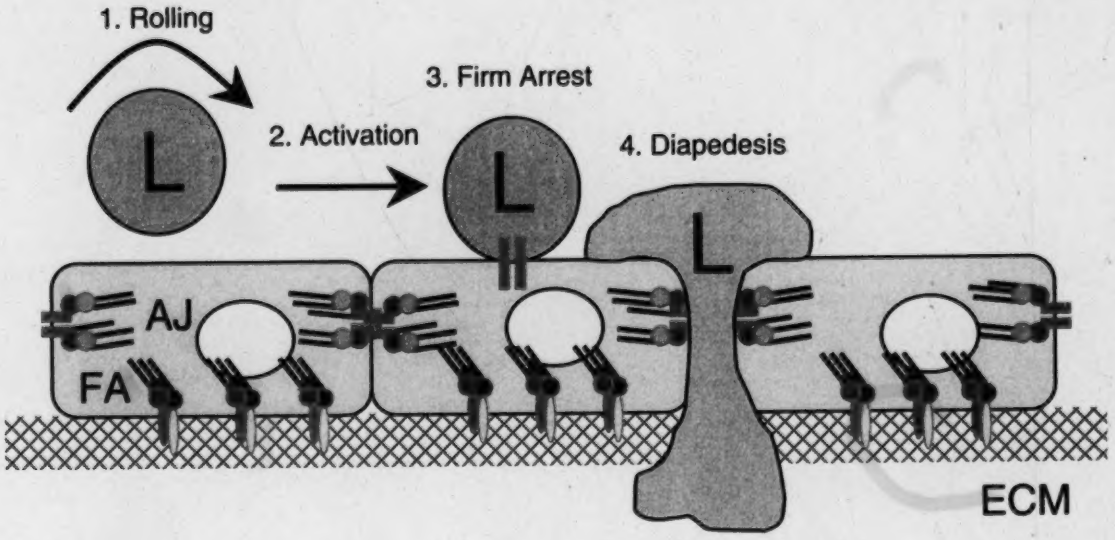


Figure 1.1. The multistep paradigm of lymphocyte transendothelial migration. Lymphocytes marginate within the blood vessel and transiently interact with the endothelium via selectin: addressin interactions (1). Chemokine-mediated lymphocyte activation (2) facilitates integrin-mediated firm adhesion (3). The paradigm concludes with lymphocyte transendothelial migration (4). AJ: adherens junction, ECM: extracellular matrix, FA: focal adhesion, L: lymphocyte.

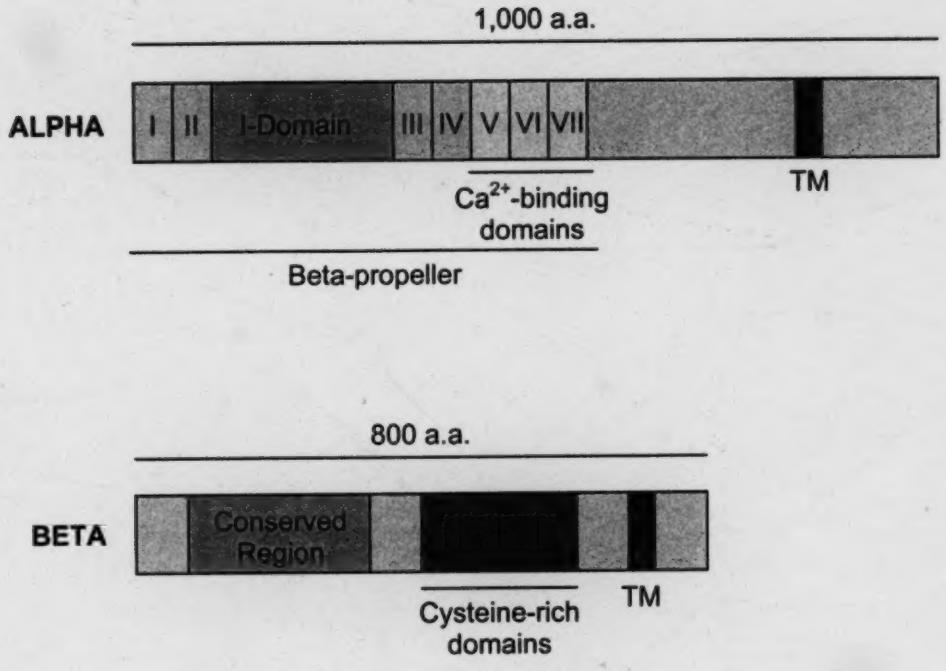


Figure 1.2. The integrin alpha and beta chains. Both chains have large N-terminal domains comprised of multiple motifs involved in ligand binding, a small transmembrane domain and C-terminal cytoplasmic tails associated with the cytoskeleton and involved in integrin activation.

Adapted from [53].

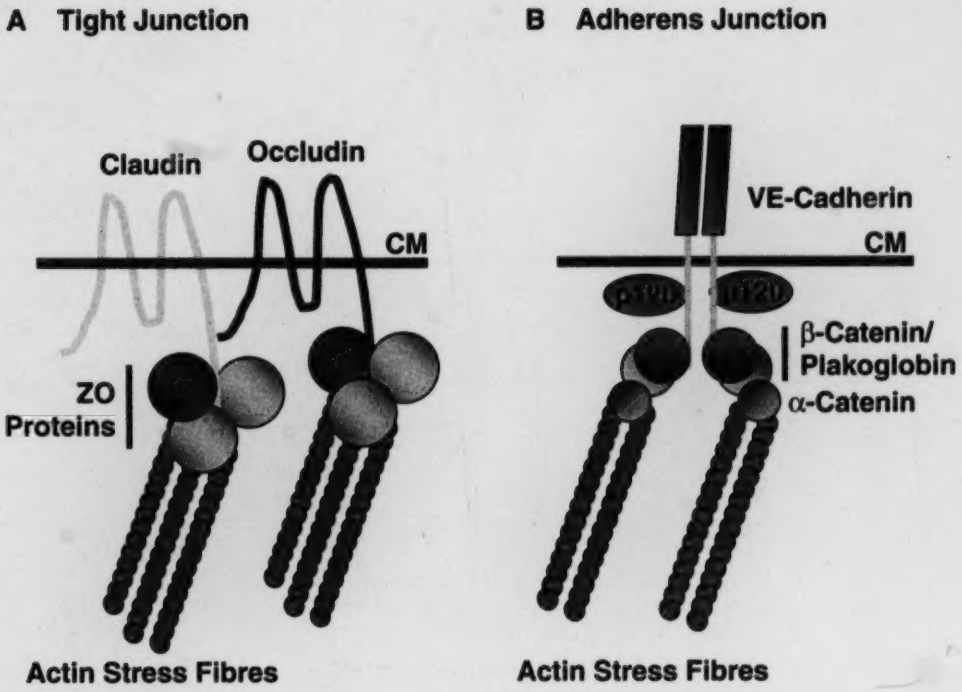


Figure 1.3. The tight junction (A) and the adherens junction (B). Both complexes contain integral membrane-spanning and cytoplasmic proteins, which interact, in turn, with the actin stress fibres of the cytoskeleton. CM: cell membrane, p120: p120^{ctn}, VE-cadherin: vascular endothelial cadherin, ZO: zonula occludens proteins.

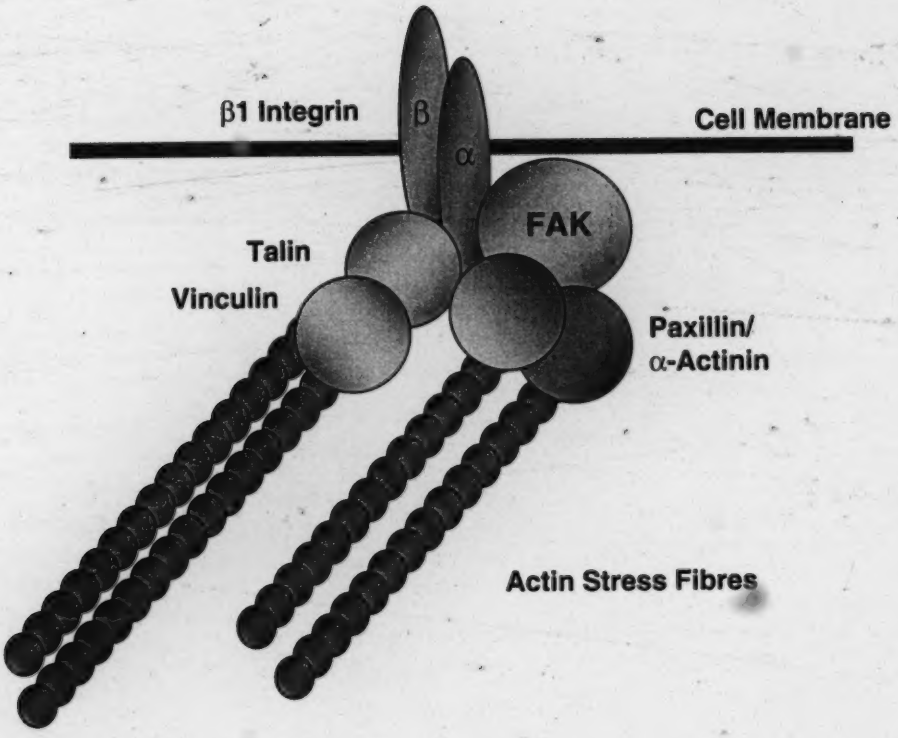


Figure 1.4. The matrix receptor (focal adhesion) complex. A transmembrane integrin heterodimer links the endothelial cell to the basement membrane. Intracellularly the integrin is linked to various structural and signalling proteins, including pp125FAK, talin, α-actinin, paxillin and vinculin. These proteins link the integrin to the stress fibres of the actin cytoskeleton. FAK: focal adhesion kinase.

CHAPTER TWO

Materials And Methods

2.1 Growth media and antibiotics

Fetal bovine serum (FBS), cell culture HBSS, DMEM, RPMI 1640 and M199 media, penicillin/streptomycin (P/S; 50 U/mL penicillin, 50 μg/mL streptomycin sulfate), glutamine (gln; 2 mM) and trypsin-ethylenediaminetetraacetic acid (0.125% trypsin, 0.5 mM EDTA in HBSS) were purchased from Life Technologies (Grand Island NY). FBS was heat inactivated and decomplemented by a 30 minute incubation at 56°C. Cell dissociation solution was from Sigma (St. Louis MO). Endothelial cell growth supplement (5 mg/mL ECGS, 10 mg/mL heparin in HBSS) was purchased from Becton Dickinson (Franklin Lakes NJ).

2.2 Solutions and buffers

Solutions were prepared using assured or tissue culture grade reagents in double-distilled deionized water (ddH_20). Dulbecco's phosphate buffered saline (D-PBS) was purchased from Life Technologies. Where D-PBS containing $CaCl_2$ was used, the solutions were prepared separately in ddH_2O and combined prior to use to prevent precipitation of $Ca_3(PO_4)_2$ crystals. Integrins require divalent cations for functionality, and thus $CaCl_2$ was used to maintain cell adhesivity.

2.3 Reagents and antibodies

Goat serum was obtained from Life Technologies. Collagenase was from Worthington Biochemical Corporation (Lakewood NJ). CellTracker green, CellTracker orange and Alexa Fluor 488-conjugated anti-FITC polyclonal antibody (pAb) were obtained from Molecular Probes (Eugene OR). Recombinant human IL-4, TNF and MCP-1 were from Peprotech Inc (Rocky Hill NJ). Monoclonal antibodies against paxillin were purchased from Transduction Labs (Mississauga ON). Anti-vinculin (hVin) mAb, phytohemagglutinin (PHA), concanamycin A, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Activating CD11a mAb (MEM-83) was from Monosan (Uden Netherlands). Negative control mouse IgG_1 and mAb to CD31 were from Dako Diagnostics (Glostrup Denmark). mAb to CD29 (TS2/16) and CD18 (TS1/18), were from ATCC (Rockville Pike MD). Anti-CD54 and anti-CD106 mAb were from Pharmingen (Mississauga ON). FITC:CD3 mAb and FITC:IgG_{2A} were from Caltag Laboratories (Burlingame CA). PE-conjugated mouse IgG_1 was from Southern Biotechnology Associates (Birmingham AL). PE:CD49d and anti-CD102 mAb were from Chemicon International (Temecula CA). FITC- and Texas Red-conjugated AffiniPure $F(Ab')_2$ fragment goat anti-mouse IgG, FITC-conjugated AffiniPure $F(Ab')_2$ fragment goat anti-rabbit IgG (H+L), horseradish peroxidase (HRP)- and biotin-SP-conjugated AffiniPure goat anti-mouse IgG (H+L) secondary antibodies were purchased from Jackson Immunoresearch (West Grove PA).

2.4 Cell culture

2.4.1 Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as described [172]. Briefly, umbilical veins were rinsed with D-PBS until perfusate was clear. Cord veins were air-purged, distally clamped, and rinsed with D-PBS. Collagenase (1 mg/mL [150-200 U/mg activity], 4-10 mL) was injected into the vein and following a 15-20 minute incubation, the EC were released by a D-PBS wash. Cells were centrifuged, resuspended and maintained in M199, supplemented with 20% FBS, P/S, gln and ECGS. Cells were stored in a humidified incubator at 37°C, with 95% humidity and 5% CO_2.

EC cultures were expanded on 2 mg gelatin-coated 75 cm^2 flasks, passaged by exposing the cells to trypsin-EDTA or cell dissociation solution for 30 seconds or 45 minutes, respectively, and quenched with M199 media. Cells were centrifuged for 10 minutes at 160 x g at 24°C in a Beckman GS-6R centrifuge equipped with a GH-3.8 rotor, replated at appropriate densities and grown to confluence. EC were used experimentally between passages 3 and 7.

2.4.2 Lymphocytes

Human peripheral blood lymphocytes (PBL) were isolated from either healthy volunteer donors or buffy coat preparations (Canadian Red Cross, Edmonton, AB), obtained under a protocol approved by the University of Alberta Health Research Ethics Board. Briefly, mononuclear leukocytes were isolated by

diluting whole blood 1:3 with HBSS and underlayering with Lymphoprep solution (Nycomed, Oslo Norway) as per the manufacturer's instruction. Cells were density-centrifuged at 525 x g for 30 minutes at 24°C without braking. The peripheral blood mononuclear cell (PBMC) population was removed and resuspended in 40 mL HBSS, and centrifuged for 15 minutes at 415 x g. Cells were washed twice more by centrifugation at 160 x g for 10 minutes in 40 mL HBSS to remove the platelet population. The PBL population was further enriched by incubating the PBMC over uncoated and 0.5 μg fibronectin-coated plastic dishes for 60 minutes at 37°C. Positively selected PBL were centrifuged for 10 minutes at 160 x g and resuspended at 1-2x10^6 cells/mL in RPMI 1640 supplemented with 10% FBS, P/S and gln. In experiments using activated lymphocytes, the PBL were treated overnight with lectin, PHA (5 μg/mL, Sigma). All PBL were treated overnight with concanamycin A (1 nM, Sigma) to inhibit cytotoxic killing. PBL were stored as in Section 2.4.1. Prior to use, PBL were fluorescently-labelled with CellTracker green (7.5 nM) or CellTracker orange (10 nM) for 30 minutes.

In experiments using resting lymphocytes, the rationale was to induce PBL adhesion to the EC without inducing classical activation of either cell type. Thus, PBL were either untreated or treated with 100 ng/mL MCP-1 at time of addition to either untreated EC or EC pre-treated for 24 hours with IL-4 (100 ng/mL). In an alternate approach, resting PBL were pre-treated for 30 minutes at 37°C with

IgG_1 or an activating mAb against CD11a, MEM-83 (2 μg/mL), in D-PBS with 1 mM $CaCl_2$ (D-PBS/$CaCl_2$; pH=7.4). The PBL were washed by centrifugation at 235 x g for 5 minutes at 24°C in D-PBS/$CaCl_2$ to remove any unbound antibody and added to untreated EC.

2.4.3 JY lymphoblasts

CD29 integrin-deficient JY human lymphoblastoid cells were a generous gift of Dr. John Wilkins (University of Manitoba, Winnipeg MN). JY cells were maintained at a density not exceeding $1x10^6$ cells/mL in RPMI 1640, supplemented with 10% FBS, P/S and gln and were expanded for 7 days prior to use (stored as in Section 2.4.1).

2.4.4 KD fibroblasts

Mer+ KD human fibroblasts were a generous gift of Dr. James Stone (University of Alberta, Edmonton AB). KD fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, P/S and gln. Cells were expanded on 2 mg gelatin-coated 75 cm^2 flasks, stored as described (Section 2.4.1) and used experimentally between passages 3 and 5.

2.5 Lymphocyte membrane preparation

Membranes from PHA-treated or freshly isolated lymphocytes were prepared as described [173]. Briefly, PBL were washed twice in ice-cold D-PBS/$CaCl_2$ and suspended on ice in 2-3 mL T cell membrane lysis buffer (0.25 M sucrose, 0.1 M

$MgCl_2$, 10 mM Tris-HCl, 250 U/mL aprotinin, and 1 mM PMSF). The PBL were sonicated for three 10 second bursts at 45 W. Subsequently, nuclei were separated by centrifugation for 15 minutes at 650 x g at 4°C. The membrane fraction was isolated from the resulting supernatant by ultracentrifugation at 100,000 x g for 30 minutes at 4°C in a Beckman Coulter TL-100 centrifuge equipped with a TLA 100.5 rotor. The isolated membranes were re-suspended in M199 and membranes were added in a minimal volume to EC (in 6- or 24-well Falcon polystyrene dishes) at the indicated cell equivalent ratios. Lymphocyte cell equivalent values were determined from PBL counts performed at the start of the protocol.

2.6 Immunocytochemistry

To assess FA, EC were cultured for 14-20 hours on 50 $\mu g/cm^2$ gelatin-coated, sterile 12 mm-diameter glass coverslips in 24-well dishes. Fluorescently-labelled effector cells (PBL, JY, or KD fibroblasts) were added to 0.4 mL EC at a ratio of 3:1-5:1 in 0.1 mL M199 and sedimented by centrifugation at 40 x g for 5 minutes.

At the indicated period of co-culture, the EC were fixed with ice-cold 2% PFA in D-PBS/$CaCl_2$ for 30 minutes. EC were washed with D-PBS/$CaCl_2$ and permeabilised with a 0.5% Triton X-100 containing buffer (20 mM Hepes, 50 mM NaCl, 3 mM $MgCl_2$ and 300 mM sucrose, pH=7.4) for 15-30 minutes. Cells were washed and non-specific binding was blocked with 10% decomplemented goat serum for 15-30 minutes. The cells were stained with mAb to either vinculin or

paxillin (1.6 μg/mL) in 4% goat serum for 40-60 minutes at room temperature. As a negative control, mouse IgG_1 was used (1.6 μg/mL). The cells were washed with 4% goat serum in D-PBS/$CaCl_2$ to remove any unbound antibody. Cells were then incubated with FITC- or Texas Red-conjugated AffiniPure $F(Ab')_2$ fragment goat anti-mouse IgG H+L secondary antibody (14 μg/mL). Coverslips were washed at least three times prior to being mounted on glass slides in Vectashield fluorescence mounting media (3 μL, Vector Labs, Burlingame CA) and Permount mounting solution (Fisher Scientific, Pittsburg PA).

In experiments where effector cells were treated with mAb prior to being added to EC, the following staining conditions were used. Cells were fixed, permeabilised and blocked as previously described. EC were stained using a FITC-conjugated mAb to paxillin (4.8 μg/mL), and an Alexa Fluor 488 conjugated anti-FITC pAb (14 μg/mL).

2.7 Fluorescence microscopy and image analysis

Images of EC FA proteins were acquired using a UV transmission fluorescence Olympus BX40 microscope (Olympus, Tokyo Japan) with a 100x objective under oil. The microscope was equipped with a modular wide-band interference blue excitation filter cube with bandpass barrier (excitation: 460-490) and a narrow band green excitation modular filter cube (excitation: 530-550). Images were taken with a Spot2 Grade 2 KAF 1400 CCD 1315 x 1033 colour digital camera

(0.6x relay lens) using the Spot Advanced Diagnostics computer program (Diagnostic Instruments, Sterling Heights MI). All parameters related to the light source and image acquisition were held constant for each session.

To eliminate bias in the selection of EC, microscope fields were selected based on the absence or presence of adherent effector cells (PBL, JY, or KD) identified in the first fluorescence channel by their CellTracker label. The fluorescence filter was then changed to the second channel and the focal plane adjusted to the base of the EC to acquire an image of the FA. Staining with mouse IgG served as a negative control. All images were photographed from the same coverslip, reducing the potential for artefacts. Images were categorised according to the absence, presence or number (1-4, >4) of EC-adherent PBL, JY or KD. All images within an experiment were photographed at the same time using the same exposures and microscope settings. Experiments were performed a minimum of three times and within each experimental group, 15 EC were photographed. Acquisition of more images was not attempted due to the concern of photobleaching.

In experiments using PBL membranes or drug treatment, a modification of the above method was utilised. Briefly, the focal plane was adjusted such that the outlines of the EC, but not the FA themselves were in focus. Following random selection of an EC, the focal plane was adjusted to the level of the FA proteins, and an image was captured. In any experiment, if the EC was too large to be

contained within the field of view, or if the FA were heavily obscured, an image was not captured.

The digital images were analysed using Image-Pro Plus software (Media Cybernetics, Silver Spring MD). Intensity calibration settings were adjusted to subtract non-specific, background staining and an area of interest was drawn around the EC FA. From this region, the sum fluorescence intensity of the paxillin or vinculin staining was calculated. In certain experiments, a second area of interest was drawn around the periphery of the EC to calculate total EC area.

2.8 Flow cytometry

Expression of EC, PBL or JY surface adhesion molecules was determined by flow cytometry. Cells were harvested and washed in D-PBS/$CaCl_2$ and suspended in primary antibody or isotype control Ig in 2-5% serum, for 30-45 minutes at 4°C. Cells were washed to remove any unbound antibody and where indirect staining was used, resuspended in FITC-conjugated AffiniPure $F(Ab')_2$ fragment goat anti-mouse IgG H+L secondary antibody (14 μg/mL) for 30-45 minutes at 4°C. Cells were washed and suspended in 2% PFA. Analysis was performed using a Becton-Dickinson (San Jose CA) FACScan using CellQuest software. Mean fluorescence intensity values were determined following data acquisition by setting M1 marker gates at 1% of isotype control stained cells.

2.9 Immunoblotting

In experiments assessing CD29 expression, EC and JY cells were washed in D-PBS/$CaCl_2$ and lysed in 0.125 mL boiling modified Laemmli sample buffer (MLSB; 2% glycerol, 1.5 M Tris-HCl [pH=6.8], 4% SDS, 1 mM PMSF, 25 U/mL aprotinin, and 0.5 mM Na_3VO_4) (modified from [40]) for 10 minutes.

In experiments assessing FA proteins, EC were grown to confluence on 6-well 1 mg gelatin-coated plastic dishes. Cells were washed in D-PBS/$CaCl_2$ and harvested in one of two ways. In experiments examining total cell protein, cells were harvested with 0.2 mL boiling MLSB. To address changes in paxillin localisation to the EC FA, the cells were treated for 60 seconds with gentle rocking with 0.2 mL cytoskeleton (CSK) buffer (10 mM Pipes [pH=6.8], 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μM Na_2MoO_4, 0.3 M sucrose, 0.5 mM Na_3VO_4, 25 U/mL aprotinin, 2 μg/mL leupeptin, 1 mM PMSF, and 0.5% Triton X-100) (modified from [34]) to remove cytosolic, but not plate-bound protein. The remaining plate-bound fraction was harvested following a 10 minute incubation with boiling MLSB with scraping.

Protein concentrations were determined using a colorimetric modified Lowry method (Bio-Rad DC protein assay, Bio-Rad, Hercules CA).

Lysates containing equal amounts of protein were resolved on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing

running buffer (0.025 M Tris, 0.192 M glycine, 0.1% sodium dodecyl sulfate, pH=8.3). Protein was transferred to PVDF membrane (Amersham Canada, Baie D'Urfe PQ) using a Bio-Rad TransBlot apparatus containing transfer buffer (25 mM Tris, 0.2 M glycine and 20% methanol). The transfer apparatus was disassembled and membranes were rinsed with TBS-Tween (TBS-T; 100 mM Tris, 0.9% NaCl, 0.1% Tween20, pH=7.5) to remove methanol. Subsequently, membranes were incubated with blocking solution containing either 4% BSA or 5% milk powder in TBS-T for 60 minutes at RT or overnight at 4°C to prevent non-specific binding of antibodies to the membrane. The membranes were then incubated with primary mAb (described in results) in 4% BSA or 5% milk powder (in TBS-T) either for 60 minutes at RT or overnight at 4°C. The membranes were washed three times with TBS-T and incubated for 60 minutes with horseradish peroxidase- or biotin-SP-conjugated AffiniPure goat anti-mouse IgG (H+L) secondary Ab in 1% goat serum with either 1% BSA or 5% milk powder at RT. Membranes were washed in TBS-T and where biotinylated secondary Ab was used, treated with Vectastain (ABC Kit, Vector Labs) for 30 minutes and washed. Membranes were enhanced with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford IL) then exposed to Biomax MR X-ray film (Eastman Kodak, Rochester NY). Times for chemiluminescent substrate incubation and film exposure were determined for individual experiments, in order to achieve an optimal, unsaturated result.

2.10 Statistical analyses

Data are expressed as mean ± SEM of *n* independent experiments. Data was analysed using SPSS software, version 10.0 (SPSS, Chicago IL). Statistical significance was determined by comparing means in a one-way analysis of variance (ANOVA) test using a Scheffe (equal variances assumed) post-hoc comparison. Statistical significance was defined as $p<0.05$.

CHAPTER THREE

Results

3.1 Optimisation of experimental conditions

3.1.1 Cell growth conditions

Prior to addressing the effect of lymphocyte (PBL) adhesion on endothelial cell (EC) abluminal matrix receptor complexes (focal adhesions, FA), experimental variables and the analysis protocol required optimisation. Lack of coverslip pre-treatment resulted in no EC adhesion. Both water bath sonication and nitric acid etching of coverslips resulted in similar high degrees of EC adhesion. An appropriate growth matrix was determined to be gelatin (as fibronectin did not further enhance EC adhesion). Optimal gelatin concentration (per coverslip), maximising EC adhesion while minimising background staining, was determined to be 50 $\mu g/cm^2$. In EC which had reached confluence, the EC FA became difficult to assess, and thus, a subconfluent plating density of 50,000 EC/coverslip was used. FA from subconfluent EC have similar morphology to those from confluent monolayers [174]. Additionally, EC plating duration (prior to fixation) did not affect or change FA morphology. Under the conditions described (elaborated in Section 2.6), EC remained adherent to the coverslips with minimal losses due to detachment throughout the duration of the staining protocol.

3.1.2 *Immunocytochemistry protocol*

Next, variables within the immunocytochemistry protocol were addressed. Permeabilisation buffer detergent concentrations (0.5%, 1% and 2% Triton X-100) yielded similar FA staining. A blocking step with 10% goat serum was added to minimise non-specific staining. Antibodies were titrated to maximise specific FA protein staining while minimising non-specific background staining.

3.1.3 *Image capture and analysis*

To validate that the image capture and analysis methods would reflect changes in the endothelial FA, EC were either untreated, or treated for 4 hours with 10, 30 or 100 μg/mL of the known tyrosine kinase inhibitor, genistein or with identical volumes of dimethyl sulfoxide (DMSO) (vehicle). Genistein has previously been shown to cause the attenuation of HUVEC FA [175] at a concentration of 100 μg/mL following 6 hours of contact. I used a reduced time of co-culture, as at the latter time point many of the EC had detached from the matrix. Following 4 hours of co-culture, the EC were fixed with 2% PFA and permeabilised using a 0.5% Triton X-100 buffer. Non-specific staining was blocked with 10% goat serum and cells were stained using an anti-paxillin mouse mAb (2 μg/mL) and a FITC-conjugated goat anti-mouse secondary Ab (14 μg/mL). To confirm specificity of staining, mouse IgG_1 (2 μg/mL) was used as a negative control.

I examined the localisation of paxillin in EC FA complexes after the various treatments. In the absence of any pre-treatment (Figure 3.1A), or when the cells were pre-treated with DMSO (the volumetric equivalent of 100 μg/mL genistein) (Figure 3.1B), large, plaque-like FA anchored the EC to the underlying matrix. In contrast, when EC were treated for 4 hours with 30 μg/mL genistein (Figure 3.1C), the EC FA were remodelled into smaller, punctate focal contacts. FA integrity was further attenuated when EC were treated with 100 μg/mL genistein (Figure 3.1D).

I quantitated the changes in EC FA assembly by determining the fluorescence intensity of staining against FA paxillin among sham-treated EC compared to that from EC pre-treated with DMSO or genistein. Sum fluorescence intensity values were calculated for DMSO and genistein-treated EC and normalised to the mean values of untreated EC. Figure 3.2 reveals that EC FA were remodelled following 4 hours of treatment with 30 μg/mL (66.8±9.5%) and 100 μg/mL (16.6±4.6%), but not 10 μg/mL (97.1±17.4%), genistein. Attenuation of FA fluorescence intensity was not observed when the EC were either untreated (100.0±9.2%) or treated with equivalent volumes of vehicle alone (DMSO) (85.9±12.0%, 109.6±11.0% and 102.8±21.8%, respectively). This confirmed that calculating the fluorescence intensity from within a selected region of interest encapsulating the endothelial FA reflected the qualitative changes in the EC FA and validates comparison between coverslips.

3.1.4 Resolving image analysis bias

As described in Section 2.6, fluorescently-labelled PBL were added to EC plated on glass coverslips and sedimented by brief centrifugation. Following co-culture for the indicated times, the cells were fixed and stained for EC FA proteins. At analysis, to ensure for the unbiased identification of EC for image capture, EC were identified for imaging based on the absence or presence of adherent PBL. Following acquisition, images were analysed by drawing a select region of interest around the EC FA and quantitating the sum fluorescence intensity within this area. However, this technique of encompassing the FA could be viewed as subjective.

To alleviate the concern of personal bias influencing the selection of the region of interest, I had an individual who was blinded to the experimental groups and expected outcome re-analyse an experimental set. The student was provided with an image analysis protocol, and 3 sets of 15 unlabelled images from a single experiment. The image sets were: EC alone, EC stimulated with membranes prepared from PHA-stimulated or freshly isolated PBL. Membranes are undetectable in images of EC FA, and thus, the groups could not be differentiated from each other based on the presence or absence of effector cells. The image analysis protocol contained a definition of focal adhesions, and the methods for region of interest selection of EC FA, and calculation of fluorescence intensity from this region. Values were then recorded. The FA fluorescence values obtained by this method were compared against values I

obtained independently. In both cases, sum fluorescence intensity values were calculated for membrane-stimulated EC and normalised to the mean values of untreated EC. As shown in Figure 3.3, there was no significant difference between the values obtained by my own (SCM) or by independent second party (KAH) analysis. In the presence of membranes prepared from PHA-treated PBL, similar changes in FA fluorescence intensity were determined by both analyses (46.5±5.0% and 58.5±6.3%, respectively). A similar result was observed following stimulation of EC with membranes prepared from freshly isolated PBL (53.9±9.6% and 59.9±10.0%, respectively). This confirmed that any bias towards selection of FA for enclosure within a region of interest was negligible and validated the analysis methods performed throughout the remainder of these studies.

3.2 Relationship between EC area and fluorescence intensity

EC of different sizes were present on the coverslips, and thus I sought to determine whether fluorescence intensity was dependent upon EC area. To address this question, I photographed 180 EC (in four sets, each set containing three individual experimental conditions) stained for FA proteins paxillin or vinculin. Images were analysed and the EC areas and FA sum fluorescence intensity values were quantified. The mode of the EC area values was determined for each experimental set (relative cell area; RCA) and raw EC area values were then expressed as % RCA. For those EC whose areas fell within 15% of the RCA (in each independent experimental condition), FA fluorescence

intensity values were averaged (relative fluorescence intensity; RFI). EC FA fluorescence intensity values within each experimental condition were then expressed as % RFI. Thus, each experimental set (containing three independent experimental conditions) had a single RCA value and three RFI values. Finally, FA fluorescence intensity values (as % RFI) were plotted against their respective cell areas (as % RCA) and a line of best fit was drawn. As shown in Figure 3.4, endothelial FA fluorescence intensity was not affected by cell area, and thus could be addressed independently.

3.3 Effect of PHA-treated PBL on endothelial FA fluorescence intensity

I sought to determine the effect of PHA-treated PBL contact on EC FA. I examined the localisation of paxillin in endothelial FA complexes after PHA-stimulated PBL were allowed to adhere to the luminal surface of the EC. In order to visualise the EC-adherent lymphocytes, the PBL were stained with membrane permeable CellTracker orange prior to addition to EC. Following co-culture, EC were stained as in Section 3.1.3. In the absence of PBL adhesion, EC formed large FA rich in paxillin (Figure 3.5A). I observed that unstimulated EC with surface-adherent PBL developed lamellipodia and appeared to spread on the coverslips (Figure 3.5B-D). In parallel to this change in morphology, the EC FA were remodelled into an increased number of small, punctate focal contacts.

I sought to determine the kinetics of FA remodelling after PBL adhesion. EC were co-cultured with PBL for 5, 15, 30 or 60 minutes following a brief

centrifugation step (5 minutes at 40 x g) to uniformly induce adhesion. Following co-culture, the EC were fixed and stained as in Section 3.1.3. I quantitated changes in endothelial FA by determining the sum fluorescence intensity of staining against paxillin among EC with adherent PBL normalised to that from slide-adjacent EC with no adherent PBL. As shown in Figure 3.6, loss of EC FA fluorescence intensity was maximal 15 minutes (57.8±4.9%) following stimulation with activated PBL, but was not present at an earlier, 5 minute (101.9±10.6%) time point. EC FA fluorescence intensity remained attenuated through the 30 and 60 minute time points (61.8±2.4% and 40.9±5.3%, respectively).

I determined whether this effect was on endothelial FA paxillin alone, or if other FA proteins would be similarly affected. EC were co-cultured with PHA-stimulated PBL for 120 minutes, then fixed and stained for FA paxillin or vinculin. As shown in Figure 3.7, in the presence of adherent PBL, EC FA incorporation of paxillin and vinculin appeared to be decreased. Furthermore, PBL contact resulted in cell spreading and lamellipodia formation. As shown in Figure 3.8, both vinculin and paxillin fluorescence intensity at the abluminal surface of the EC were decreased following the adhesion of 1-4 PBL (59.7±25.9% and 46.6±12.7%, respectively). Fluorescence intensity of FA vinculin (37.1±14.6%) and paxillin (36.2±15.2%) were similarly decreased in the presence of >4 adherent PBL.

3.4 β1 integrin-dependent effect of freshly isolated PBL on endothelial FA fluorescence intensity

In the previous sections, I addressed the effect of PHA-stimulated PBL adhesion on endothelial FA fluorescence intensity. Next, I sought to determine whether attenuation of EC FA fluorescence was dependent on the activation state of the PBL. Since freshly isolated PBL do not stably adhere to cultured EC, I modified the experimental design to induce sustained adhesion of the PBL to EC in the absence of PHA activation. Tight binding between PBL and EC during lymphocyte transendothelial migration is primarily integrin-mediated [62;176]; hence I exploited these adhesive interactions in my approaches.

In my first approach, I exploited interactions between the endothelial adhesion molecule CD106 (VCAM-1) and the PBL integrin, CD49d/CD29 (VLA-4; α4β1) as a model of lymphocyte trafficking to post-capillary venules *in vivo*. Resting EC were treated for 24 hours with interleukin-4 (IL-4) to induce surface expression of CD106 [96]. IL-4 was used in these experiments as, unlike TNF, it does not induce a morphological change in the EC. The ligand for CD106, CD49d/CD29, is expressed by resting memory PBL [177] and can be activated by MCP-1 treatment [62]. MCP-1-treated PBL were then added to untreated or IL-4-treated EC and allowed to adhere.

3.4.1 Endothelial surface expression of CD106

To induce surface expression of the endothelial adhesion molecule CD106, resting EC were either untreated or treated with IL-4 (100 ng/mL) or TNF (80 U/mL) for 24 hours. EC were harvested with trypsin-EDTA and suspended in D-PBS. Within each treatment group, half of the EC were stained with anti-CD106 mAb (5 μg/mL), and the remaining cells were stained using isotype control mouse IgG (5 μg/mL). A FITC-conjugated goat anti-mouse secondary Ab (14 μg/mL) was used on all cells. Antibodies were added in the presence of 2-5% goat serum to minimise non-specific binding. Cells were fixed in 2% PFA and analysed by flow cytometry. M1 gates were set at ~1% of untreated, isotype control-stained EC, and applied to all other groups. As shown in Figure 3.9A, few untreated EC expressed CD106 (16.7%). However, when the EC were treated for 24 hours with recombinant human IL-4 (Figure 3.9B), 57.2% were CD106 positive. As a positive control, EC were treated with TNF which also induced the surface upregulation of CD106 (76.1%, Figure 3.9C).

3.4.2 Lymphocyte surface expression of CD49d and CD29

The ligand for endothelial CD106 on PBL is the integrin heterodimer, CD49d/CD29. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and panned against plastic for 60 minutes to isolate the PBL population. Cells were washed and stained in the presence of 5% pooled human serum to reduce non-specific binding. As a negative control, PBL were stained using directly-conjugated FITC:IgG_{2A} or PE:IgG_1 alone or in combination. To

confirm expression of α4 integrin by PBL, cells were stained using FITC:CD3 and PE:CD49d alone or in combination. Flow cytometer instrument settings were determined from populations of unstained and isotype-stained PBL. As shown in Figure 3.10, 74.3% of the population of cells were CD3 positive, and thus, were T-lymphocytes (B), 96.2% of the cells were positive for CD49d (C), and 70.2% of the PBL were CD3+ CD49d+ (D).

PBL expression of the β1 integrin was confirmed using isotype control Ig (5 μg/mL) or the anti-CD29 mAb, TS2/16 (1:100 dilution of ascites), and a FITC-conjugated goat anti-mouse secondary Ab (14 μg/mL). PBL were analysed by flow cytometry as in Section 3.4.1. As shown in Figure 3.11, 95.23% of freshly isolated PBL were CD29 positive.

3.4.3 Effect of IL-4 and MCP-1 on endothelial FA

Prior to assessing the effect of resting lymphocyte adhesion on FA fluorescence intensity, I determined the effect of IL-4 or MCP-1 alone or in combination on the EC FA. EC were treated for 24 hours with IL-4 and for 2 hours with MCP-1 (the same amount of time the MCP-1 treated PBL would be in contact with the EC prior to fixation). Cells were fixed and stained as in Section 3.1.3. Changes in EC FA fluorescence were assessed by quantifying the intensity of staining for paxillin among EC treated with MCP-1 and IL-4 alone or together compared to that from sham-treated EC. As illustrated in Figure 3.12, neither IL-4 nor MCP-1

alone or in combination resulted in a change in EC FA fluorescence intensity (106.7±9.6%, 122.9±14.6% and 92.7±10.7%, respectively).

3.4.4 Effect of lymphocyte adhesion via CD49d/CD29:CD106 on endothelial FA fluorescence intensity

I addressed the effect of freshly isolated PBL adhesion to EC on the fluorescence intensity of the FA. Freshly isolated PBL were co-cultured with untreated or IL-4-treated EC in the presence of MCP-1 for 2 hours following a brief centrifugation step. Cells were then fixed and stained for FA paxillin as in Section 3.1.3, and analysed as in Figure 3.2. When PBL were added to EC in the absence of any pre-treatment, PBL did not adhere to the EC in a sustained manner and changes in FA fluorescence could not be assessed. However, as shown in Figure 3.13, when freshly isolated PBL were treated with MCP-1 prior to addition to unstimulated EC, some EC had adherent PBL and among those EC, FA fluorescence intensity was decreased (65.9±14.4%) compared with slide-adjacent EC with no adherent PBL. Additionally, when MCP-1-treated PBL were added to IL-4-stimulated EC, a high percentage of the EC had adherent PBL, and FA attenuation was marked (46.0±6.8%).

I sought to determine if freshly isolated PBL would elicit changes in endothelial FA fluorescence intensity with similar kinetics to their PHA-stimulated counterparts. I examined EC FA paxillin fluorescence intensity following 5, 15, 30 or 60 minutes of contact with freshly isolated PBL. As shown in Figure 3.14,

when MCP-1 treated resting PBL were added to IL-4 stimulated EC (as in Figure 3.13), FA fluorescence intensity was significantly decreased following 15 (60.9±4.7%), but not 5 (103.7±4.5%), minutes of co-culture. EC FA fluorescence intensity remained decreased through the 30 and 60 minute time points (50.7±12.0% and 53.5±2.7%, respectively).

3.5 β2 integrin-dependent effect of freshly isolated PBL on endothelial FA fluorescence intensity

In my previous approach, I exploited interactions between endothelial CD106 and PBL CD49d/CD29 to determine whether sustained adhesion of freshly isolated PBL would result in a change in EC FA paxillin fluorescence intensity. To confirm these observations without manipulating the EC, freshly isolated PBL were pre-treated with the activating mAb against CD11a (MEM-83) and added to untreated EC, which express the ligands for CD11a/CD18, CD54 and CD102.

3.5.1 Endothelial surface expression of CD54 and CD102

To confirm expression of the surface adhesion molecules CD54 and CD102, resting EC were harvested with trypsin-EDTA and suspended in D-PBS. EC were stained using isotype control IgG (5 μg/mL) or mAb against CD54, CD102 or CD31 as a positive control (5 μg/mL) and FITC-conjugated secondary Ab (14 μg/mL). All antibodies were added in the presence of 2% goat serum to minimise non-specific binding. Cells were fixed in 2% PFA and analysed as in Section

3.4.1. As shown in Figure 3.15, 80.5% of the EC expressed CD54 (A), 99.9% expressed CD102 (B) and 98.6% expressed CD31 (C).

3.5.2 Lymphocyte surface expression of CD11a and CD18

The ligand for endothelial CD54 and CD102 on PBL is the integrin heterodimer, CD11a/CD18 ($\alpha L\beta 2$). PBL were prepared and analysed as in Section 3.4.1. To confirm PBL αL integrin chain expression, anti-CD11a mAb, MEM-83 (2 μg/mL), was used. To confirm expression of the PBL β2 integrin chain, cells were stained using anti-CD18 mAb, TS1/18 (1:100 dilution of purified ascites). As a negative control, PBL were stained using isotype control Ig (2 μg/mL). As shown in Figure 3.16, 99.8% of the PBL were CD11a+ (A) and 99.9% were CD18+ (B).

3.5.3 Effect of lymphocyte adhesion via CD11a/CD18:CD54/CD102 on endothelial FA fluorescence intensity

Finally, I assessed the effect of freshly isolated PBL adhesion to EC on the fluorescence intensity of the FA. Freshly isolated PBL were pre-treated for 30 minutes with control Ig or anti-CD11a mAb MEM-83 (2 μg/mL) and added to untreated EC for 2 hours following a brief centrifugation step. Cells were fixed, stained using a direct conjugated FITC:paxillin mAb (5 μg/mL) and an Alexa Fluor 488:FITC pAb (15 μg/mL) and analysed as in Figure 3.2. When untreated or control Ig-treated PBL were added to untreated EC, PBL did not adhere in a sustained manner, hence, changes in the EC FA fluorescence were not

addressed. However, as shown in Figure 3.17, when resting PBL were pre-treated with activating anti-CD11a mAb (MEM-83) and added to untreated EC, PBL adhered to EC in a sustained manner. In the presence of 1-4 adherent PBL, there was a significant decrease in EC FA fluorescence intensity (42.9±10.5%) which was further enhanced in the presence of >4 bound PBL (30.1±11.2%).

3.6 Effect of CD29-deficient JY lymphoblasts on endothelial FA fluorescence intensity

In my last set of experiments, I observed that CD11a/CD18:CD54/CD102-dependent adhesion was sufficient to induce decreased paxillin incorporation into FA, reflected by a change in endothelial FA fluorescence intensity. As resting EC did not express high levels of CD106 (Figure 3.9A), I assessed the involvement of PBL CD29 in the induction of EC FA remodelling using the CD29-deficient JY lymphoblastoid cell line.

3.6.1 JY lymphoblast surface expression of CD29

JY lymphoblast surface expression of CD29 was assessed by flow cytometry. JY cells were stained using an isotype control IgG (5 μg/mL) or mAb against CD29, TS2/16 (1:100 dilution of ascites), or as positive control, anti-CD18 mAb, TS1/18 (1:100 dilution of purified ascites). As a further control, EC were stained for CD29 expression using mAb TS2/16 at the same concentration. All antibodies were added in the presence of 2% pooled human serum to minimise non-specific binding. Cells were fixed and analysed as in Section 3.4.1. As shown in Figure

3.18A, 99.6% of the EC expressed CD29. However, only 5.5% of JY cells were CD29 positive (Figure 3.18B), although 100.0% of JY expressed CD18 (Figure 3.18C).

To confirm these observations using an alternate approach, JY CD29 expression was assessed by immunoblot. EC and JY were lysed in modified Laemmli sample buffer (MLSB; Section 2.9) for 10 minutes. Protein concentrations were determined by a colorimetric modified Lowry assay, and lysates containing 10 μg protein were resolved on a 7.5% SDS-PAGE. Protein was transferred to PVDF membrane overnight at 30V (~50 mA) at 4°C. Following a blocking step in 4% BSA, membranes were stained using a 1:1000 dilution of TS2/16 mAb (in ascites) in 4% BSA and 0.2 μg/mL biotin-SP-conjugated AffiniPure goat anti-mouse IgG (H+L) secondary Ab in 1% goat serum with 1% BSA at RT. As shown in Figure 3.19, EC (lane 1), but not JY lymphoblasts (lane 2), expressed the β1 integrin.

3.6.2 Effect of JY lymphoblasts on endothelial FA fluorescence intensity

I sought to determine the effect of CD29-deficient JY lymphoblast adhesion on endothelial FA fluorescence intensity. JY cells were stained using CellTracker orange for 30 minutes prior to addition to EC. Following a brief centrifugation step, cells were co-cultured for 60 minutes, then fixed, stained as in Section 3.1.3 and analysed as in Figure 3.2. As shown in Figure 3.20, adhesion of >1 JY

lymphoblasts to EC induced a significant decrease in EC FA fluorescence intensity (46.2±12.6%).

3.7 Effect of Mer+ KD fibroblasts on endothelial FA fluorescence intensity

Next, I studied the effect of adherent human fibroblasts (KD) on endothelial FA fluorescence intensity. KD fibroblasts, or PBL as a positive control, were added to EC (in cell equivalent ratios of 2:1 and 3:1, respectively), centrifuged briefly and co-cultured for 60 minutes. EC were fixed and stained as in Section 3.1.3 and analysed as in Figure 3.2. KD fibroblasts adhered to the EC in a sustained manner and did not require any pre-treatment. As shown in Figure 3.21, when KD fibroblasts were added to EC, EC FA fluorescence intensity was not significantly altered (130.6±12.9%) when compared to EC with no adherent KD cells (100.0%).

3.8 Effect of PBL membranes on endothelial FA fluorescence intensity

Decreases in endothelial FA fluorescence intensity are dependent upon sustained PBL adhesion. I sought to determine whether PBL membranes would be sufficient to induce loss of endothelial FA fluorescence intensity. Membranes prepared from PHA-treated PBL (PHA-PBL) or freshly isolated PBL (PBL) were added to EC at indicated cell equivalent ratios and co-cultured for 120 minutes. Cell equivalent volumes were determined from PBL counts performed prior to membrane preparation, however, the degree of adhesion to the EC could not be

determined. At the end of the co-culture period, EC were fixed and stained using mAb against FA proteins paxillin or vinculin and FITC- or Texas Red-conjugated goat anti-mouse IgG. Sum fluorescence intensities were calculated for membrane-treated EC and normalised to the mean values of mock-treated EC. Although the proportion of the PBL membranes which had adhered to the EC could not be calculated, as shown in Figure 3.22, the addition of PHA-stimulated lymphocyte membranes in PBL:EC ratios of 0.3:1, 1:1, 3:1 and 10:1 resulted in decreases in both FA vinculin (38.7±4.7%, 43.6±9.8%, 43.4±3.1% and 41.7±12.1%, respectively) and FA paxillin (55.0±6.7%, 62.7±23.8%, 68.6±11.5% and 64.3±4.3%, respectively) fluorescence intensities. I observed a similar decline in endothelial FA fluorescence intensity when membranes prepared from freshly isolated PBL were added to EC in the same cell equivalent ratios (Figure 3.23). FA vinculin fluorescence was decreased to 45.1±13.2%, 31.7±6.4%, 25.9±8.1% and 25.7±6.0%, respectively. A similar attenuation was observed when EC FA were stained for paxillin (50.9±8.3%, 56.2±14.7%, 50.6±3.1%, 46.7±7.0%, respectively). No change was observed in EC von Willebrand factor fluorescence intensity following stimulation with PBL membranes.

3.9 Biochemical confirmation of PBL-induced changes in endothelial FA

3.9.1 Optimisation of experimental conditions

Prior to addressing the effect of PBL adhesion on EC abluminal matrix receptor complexes, lysis conditions required optimisation. In the first approach, cells

were lysed with boiling modified Laemmli sample buffer (MLSB), which was sufficient to remove both cytosolic and plate-bound proteins. This was confirmed by the absence of marked amounts of protein present in a second MLSB wash. The second lysis approach, to first remove only cytosolic proteins, and then remove plate-bound protein, required optimisation. Detergent conditions in the CSK buffer were selected to minimise loss of cytoskeleton-associated (FA complex) protein. The permeabilisation buffer used in immunocytochemical studies did not appear to disrupt FA complex-associated proteins, and thus, identical detergent conditions were used in the CSK buffer. The number and duration of CSK washes were also optimised. A single treatment of the EC with CSK buffer for 60 seconds was sufficient to remove approximately half of the protein in the well (measured by protein determination). Cells were subsequently treated with boiling MLSB to remove plate-bound proteins (Section 2.9).

3.9.2 Biochemical confirmation of lymphocyte-induced changes in endothelial FA

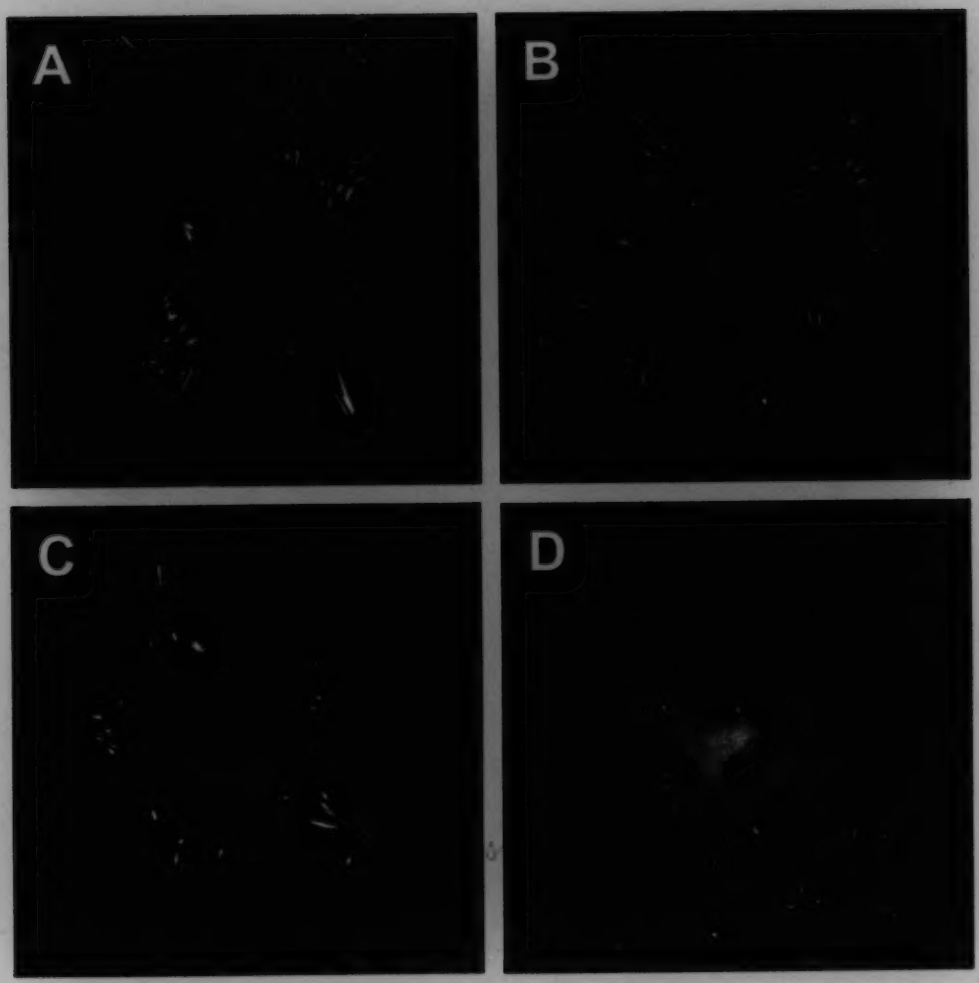
I sought to determine if I could validate my earlier observation of PBL-induced changes in endothelial FA using a biochemical approach. EC were cultured to confluence on gelatin-coated (1 mg) 6-well plastic dishes. Membranes prepared from freshly isolated PBL were added to the EC at a cell equivalent ratio of 5:1 for 60 minutes. PBL membranes contain minimal paxillin, and were used to minimise contamination of the immunoblot results with whole cell PBL paxillin. Cells were washed in D-PBS/$CaCl_2$ and lysed using either boiling MLSB alone or a combination of CSK buffer, to remove the cytosolic fraction, and boiling MLSB

to remove plate-associated proteins (Section 2.9). Protein concentrations were determined by a colorimetric modified Lowry assay, and lysates containing 2.5 μg protein were resolved on a 7.5% SDS-PAGE. Protein was transferred to PVDF membrane for 90 minutes at 100V at 4°C. Following a blocking step in 5% milk powder in TBS-Tween, membranes were stained using 25 ng/mL anti-paxillin mAb in 5% milk powder and $8x10^{-2}$ μg/mL HRP-conjugated AffiniPure goat anti-mouse IgG (H+L) secondary Ab in 1% goat serum with 5% milk powder at RT.

The results consistently showed that untreated EC and EC stimulated with PBL membranes contained similar levels of total cell paxillin (Figure 3.24, lanes 1 and 2). Furthermore, no degradation products of paxillin appeared following stimulation of EC with PBL membranes. When differential detergent solubilisation conditions were used, and cytoskeleton-associated paxillin levels were assessed, the results showed an obvious decrease in paxillin in EC stimulated with PBL membranes (Figure 3.24, lane 4) compared with unstimulated EC (Figure 3.24, lane 3). Although degradation products appeared to be present in the soluble fraction of the lysates, these were unchanged in the presence of PBL membranes (Figure 3.24, lane 6) compared with unstimulated EC (Figure 3.24, lane 5).

3.10 Figures

Figure 3.1. Photomicrographs of EC stained for FA paxillin following sham, DMSO or genistein treatment. EC were plated for 16-18 hours, sham-treated (A) or stimulated with DMSO at the equivalent volume of 100 μg/mL drug (B), 30 μg/mL genistein (C) or 100 μg/mL genistein (D) for 4 hours. Images were taken using a 100x objective under oil with a Spot2 Grade 2 KAF 1400 CCD 1315 x 1033 colour digital camera (0.6x relay lens). Images are representative of 2 independent experiments.


A
B
C
D

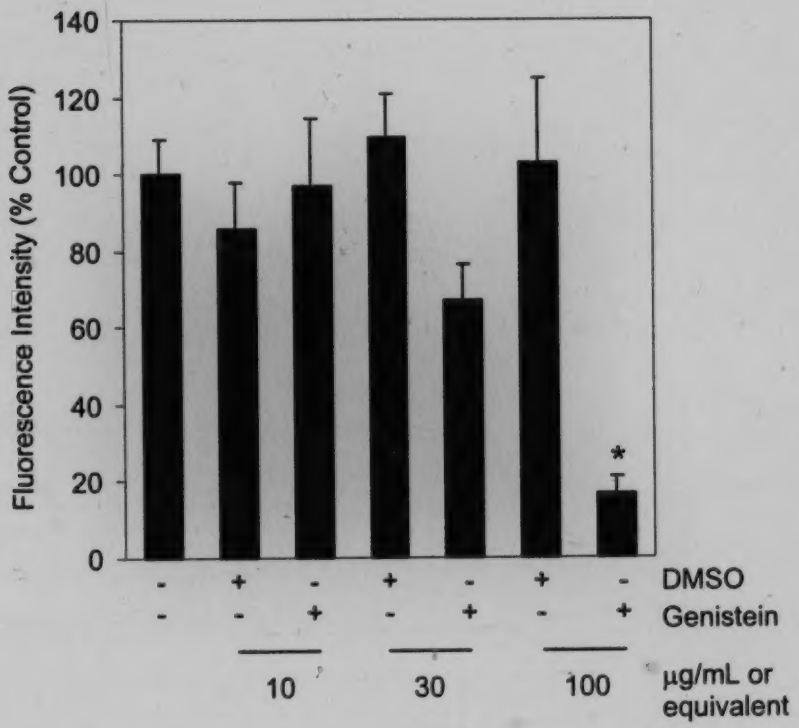


Figure 3.2. Effect of sham, DMSO or genistein treatment on endothelial FA fluorescence intensity. EC were untreated or stimulated with 10, 30 or 100 µg/mL of genistein or equivalent volumes of DMSO for 4 hours then fixed and stained for paxillin. Sum cell FA fluorescence intensity was calculated for DMSO- and genistein-treated EC and normalised to the mean values of sham-treated EC. Data are representative of 2 independent experiments, each containing 15 EC/group (mean ± SEM, *$p<0.05$ by ANOVA).

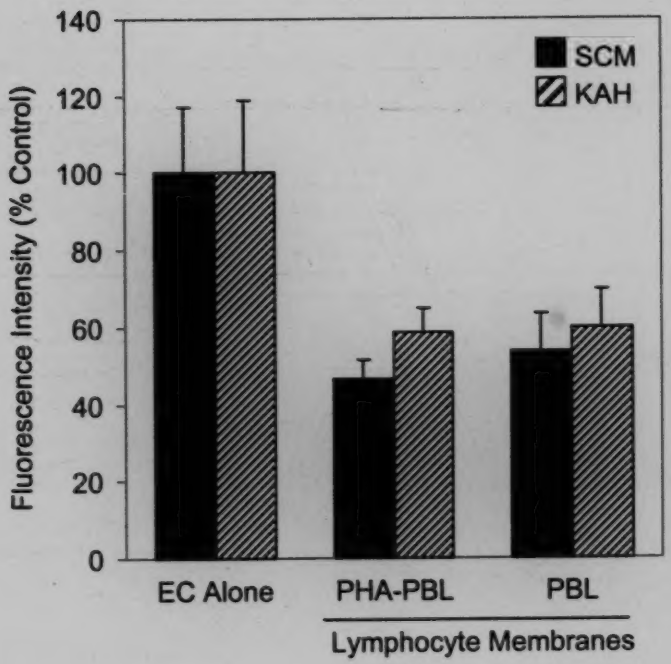


Figure 3.3. Comparison of endothelial FA fluorescence intensity values obtained following contact with membranes prepared from PHA-treated PBL (PHA-PBL) or freshly isolated lymphocytes (PBL). Sum cell FA fluorescence intensity was calculated by the experimenter (SCM) or by an independent second party (KAH) for membrane-stimulated EC and normalised to the mean values of sham-treated EC. Data are representative of a single re-analysed experiment with 15 EC/group (mean ± SEM).

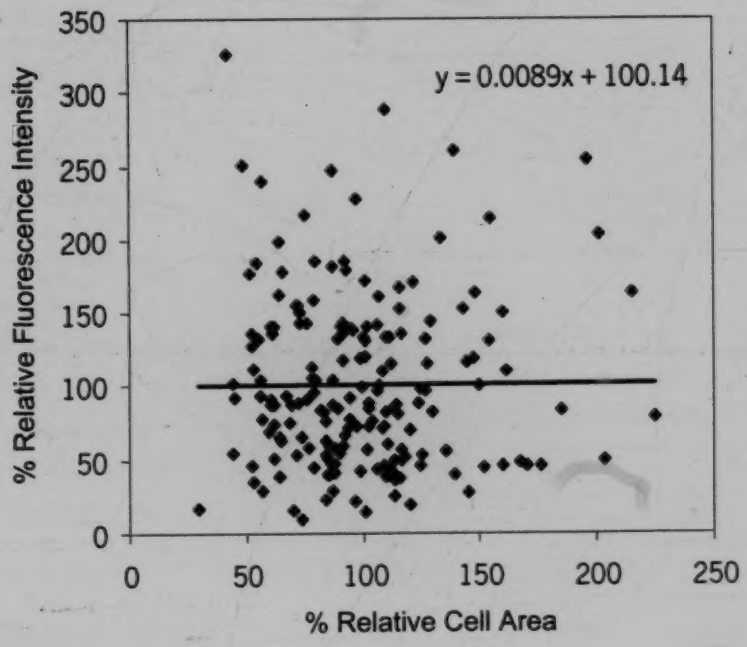
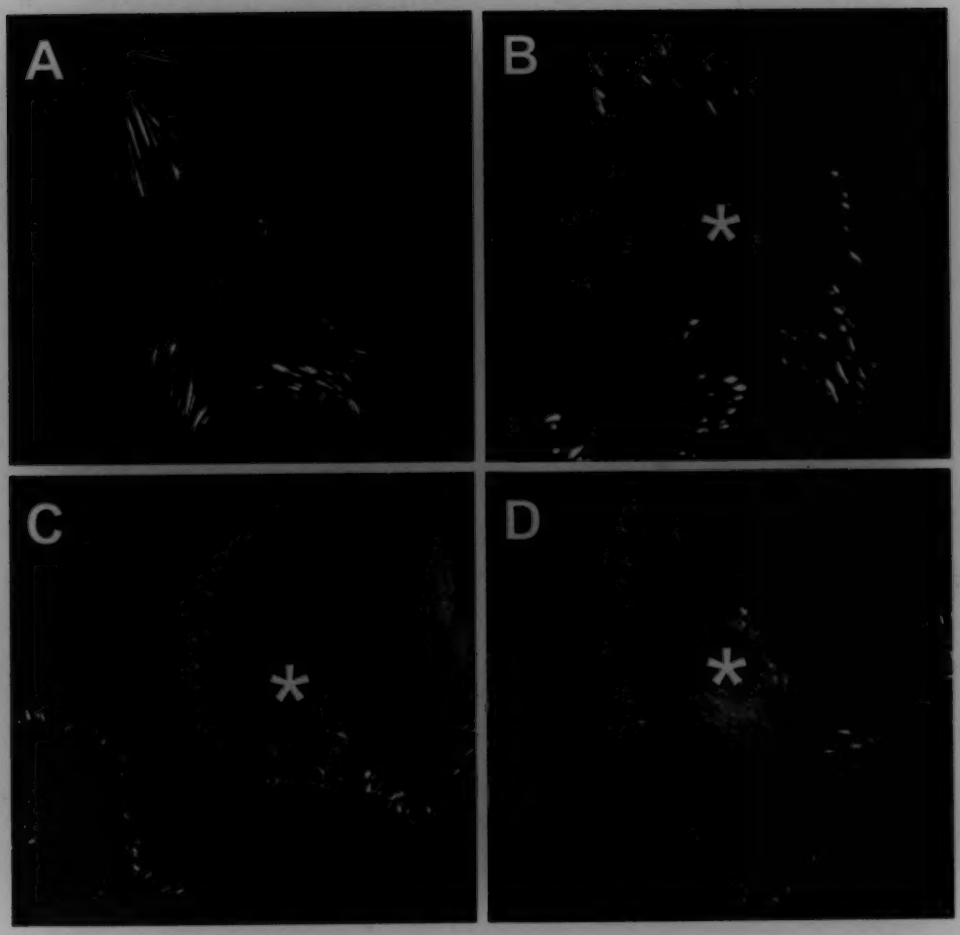


Figure 3.4. Relationship between cell area and FA fluorescence intensity of individual EC. Fluorescence intensity was determined from immunostaining for EC FA paxillin or vinculin. Data points were calculated as described (Section 3.2). Data represents 180 images from 4 experimental sets, each containing 3 independent experimental conditions.

Figure 3.5. Photomicrographs of EC stained for FA paxillin following sustained adhesion of PHA-stimulated PBL. EC were co-cultured with PHA-treated, CellTracker orange-stained PBL. EC with no adherent PBL (A) or EC with adherent PBL (marked with *) for 5 (B), 15 (C) or 30 (D) minutes. Images were taken using a 100x objective under oil with a Spot2 Grade 2 KAF 1400 CCD 1315 x 1033 colour digital camera (0.6x relay lens). Images are representative of greater than 3 independent experiments.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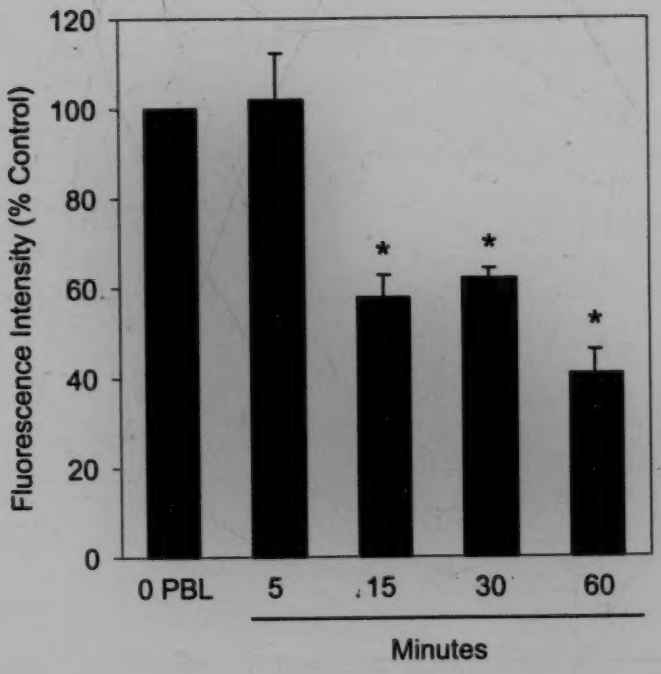
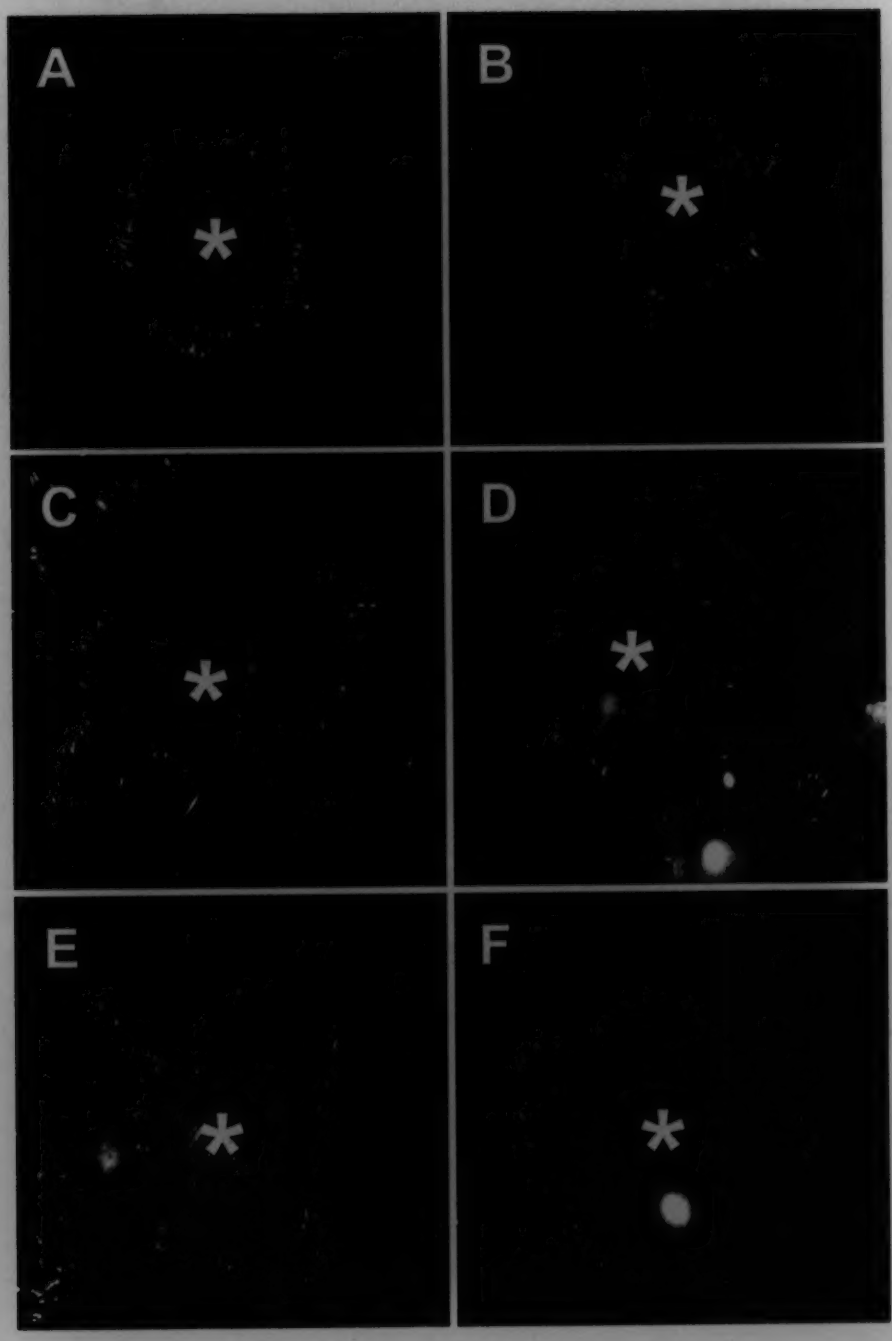


Figure 3.6. Effect of PHA-treated PBL adhesion on endothelial FA fluorescence intensity. EC were co-cultured with PHA-stimulated PBL for 5, 15, 30 or 60 minutes. Sum cell FA paxillin fluorescence intensity was calculated for PBL-adherent EC and normalised to the mean values of slide-adjacent EC with no adherent PBL. Data represent mean ± SEM of greater than 3 independent experiments, each with 15 EC/group (*$p<0.05$ by ANOVA).

Figure 3.7. Photomicrographs of EC following adhesion of increasing numbers of PHA-treated PBL. EC were co-cultured with PHA-stimulated, CellTracker green- or orange-stained PBL for 120 minutes, then fixed and stained for FA proteins paxillin (A,C,E) and vinculin (B,D,F). EC on the same coverslip (marked with *) were grouped according to the number of adherent PBL (0 [A, B], 1-4 [C, D], >4 [E, F]). Images were taken using a 100x objective under oil with a Spot2 Grade 2 KAF 1400 CCD 1315 x 1033 colour digital camera (0.6x relay lens). Images are representative of 3 independent experiments.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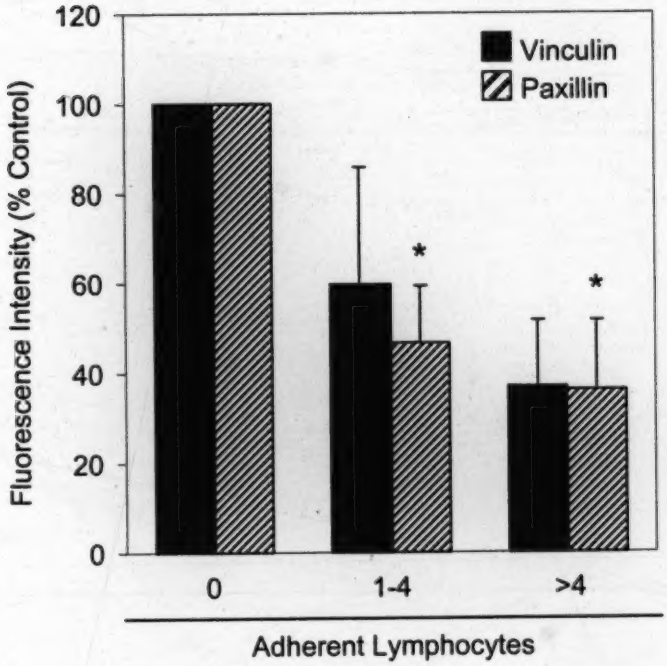

Figure 3.8. Effect of PHA-treated PBL adhesion on endothelial FA fluorescence intensity. EC were co-cultured with PHA-stimulated PBL for 120 minutes, then fixed and immunostained for FA paxillin or vinculin. EC on the same coverslip were grouped according to the number of adherent PBL (0, 1-4, >4). Images were analysed as in Figure 3.6. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group (*$p<0.05$ by ANOVA).

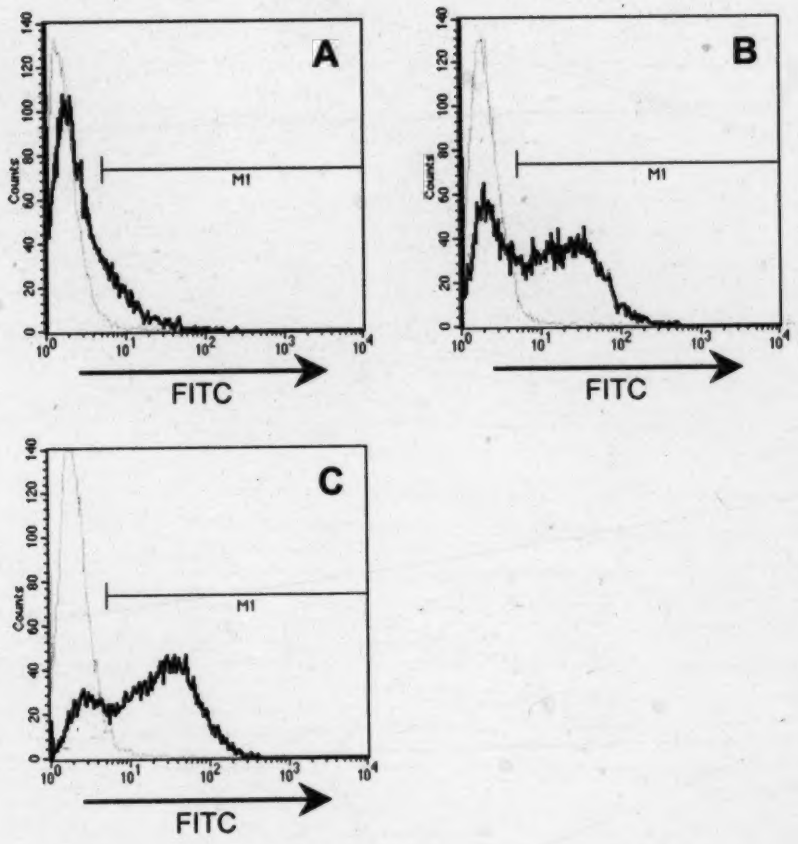

Figure 3.9. Surface expression of EC CD106 following sham, IL-4 or TNF treatment. EC were either untreated (A) or plated in the presence of 100 ng/mL IL-4 (B) or 80 U/mL TNF (C) for 24 hours. Cells were harvested using trypsin-EDTA and stained using either isotype control IgG_1 (5 μg/mL, grey line) or a mAb against CD106 (5 μg/mL, black line) and a FITC-conjugated secondary Ab. EC were washed and fixed in 2% PFA and analysed on a Becton Dickinson FACScan flow cytometer. M1 marker gates were set at 1% of isotype control stained cells. Data are representative of greater than 3 independent experiments.

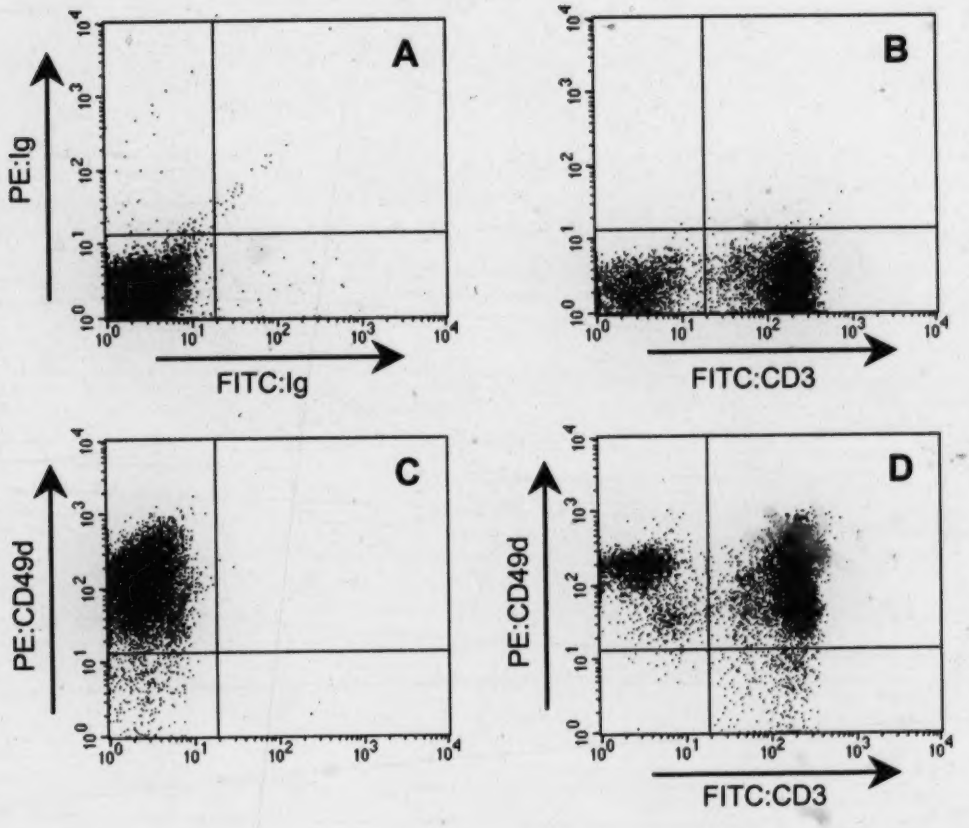

Figure 3.10. Surface expression of lymphocyte CD3 and CD49d. Freshly isolated PBL were stained using isotype control FITC:Ig and PE:Ig in combination (A), FITC:CD3 or PE:CD49d alone (B, C) or in combination (D). Cells were washed and fixed in 2% PFA and analysed on a Becton Dickinson FACScan flow cytometer. Data are representative of 2 independent experiments.

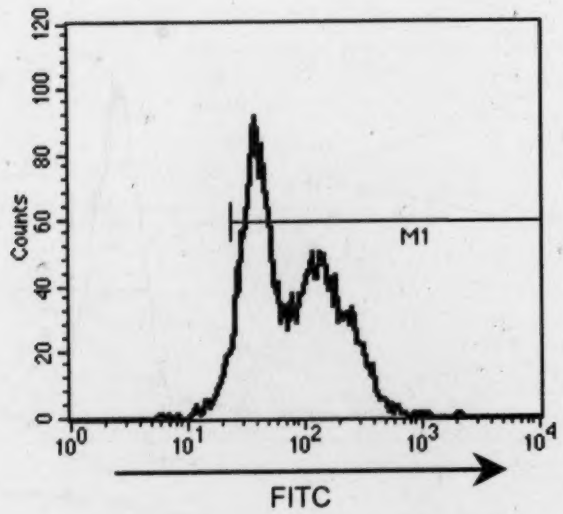

Figure 3.11. Surface expression of lymphocyte CD29. Freshly isolated PBL were stained using isotype control IgG_1 (5 μg/mL, grey line) or anti-CD29 mAb (1:100 dilution of ascites, black line) and a FITC-conjugated secondary Ab. Cells were washed, fixed in 2% PFA and analysed as in Figure 3.9. Data are representative of 2 independent experiments.

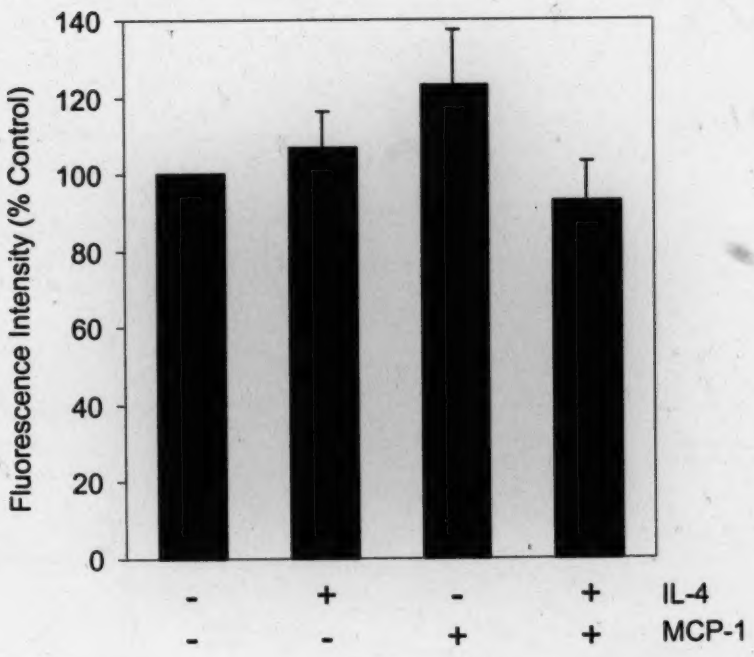

Figure 3.12. Effect of IL-4 and/or MCP-1 treatments on endothelial FA fluorescence intensity. EC were plated for 24 hours in the presence or absence of 100 ng/mL IL-4 then untreated or treated for 2 hours with 100 ng/mL MCP-1. Cells were then fixed and immunostained for FA paxillin. Sum cell FA fluorescence intensity was calculated for IL-4 and/or MCP-1-treated EC and normalised to the mean values of sham-treated EC. Data are representative of 3 independent experiments, each with 15 EC/group (mean ± SEM).

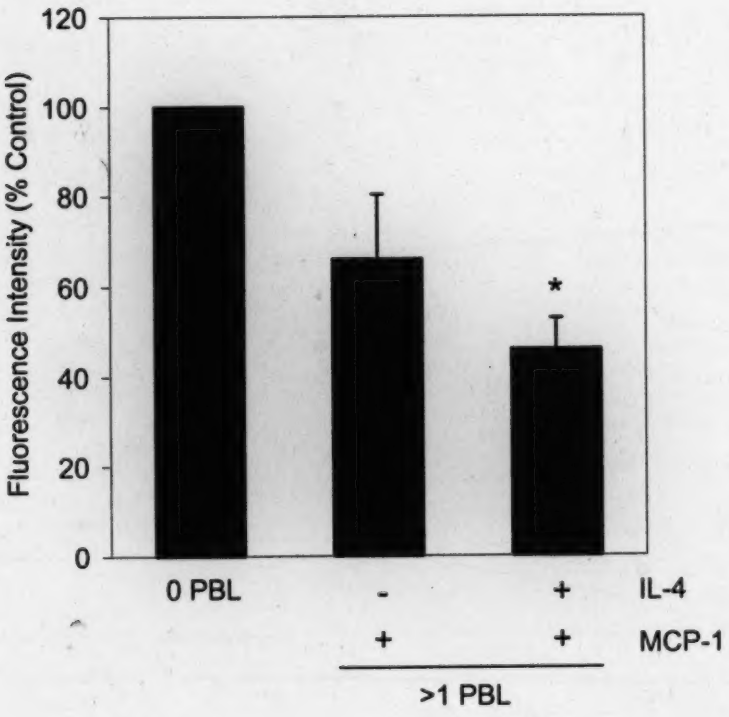

Figure 3.13. Effect of freshly isolated lymphocyte adhesion via CD49d/CD29: CD106 on endothelial FA fluorescence intensity. PBL were added to untreated EC or IL-4-treated EC in the presence of MCP-1 for 2 hours. Following co-culture, EC were fixed, immunostained for paxillin and analysed as in Figure 3.6. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group (*$p<0.05$ by ANOVA).

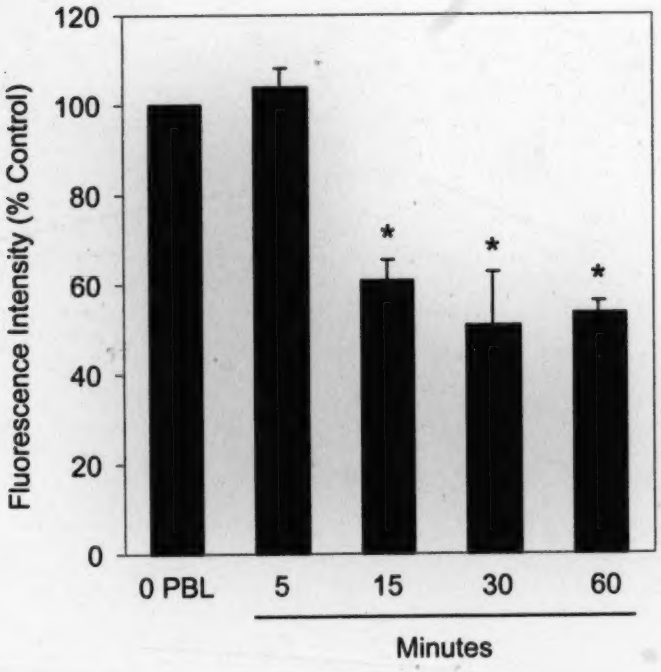

Figure 3.14. Effect of freshly isolated lymphocyte adhesion on endothelial FA fluorescence intensity. PBL were added to untreated EC or IL-4-treated EC in the presence of MCP-1 for 5, 15, 30 or 60 minutes. Images of FA paxillin-stained EC with or without adherent PBL were analysed as in Figure 3.6. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group (*$p<0.05$ by ANOVA).

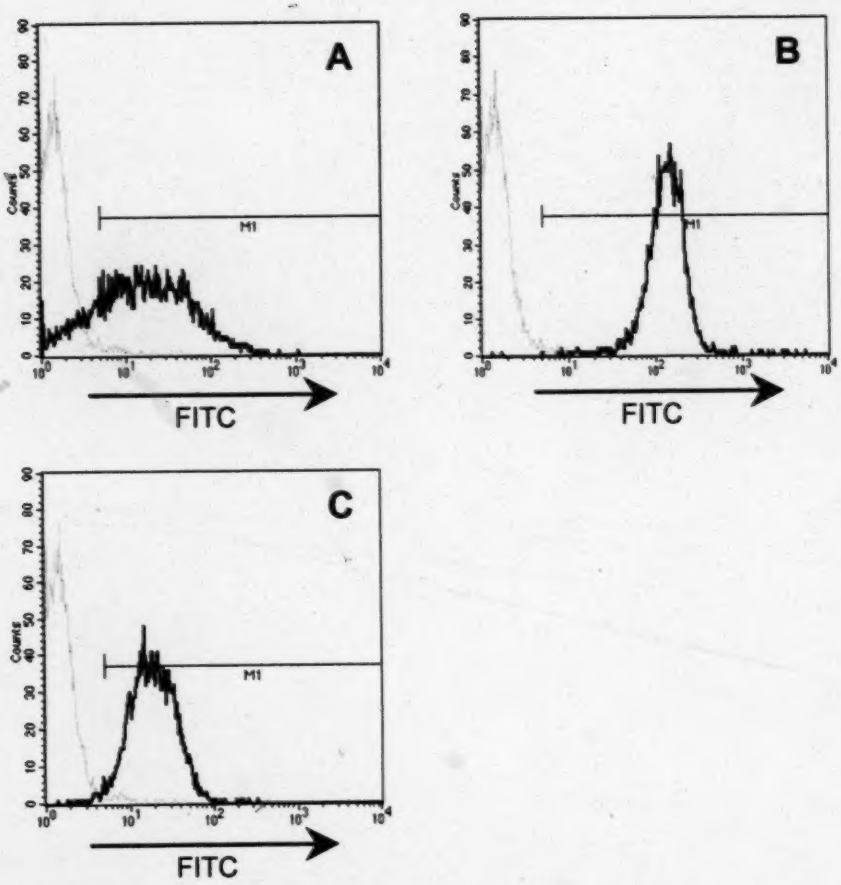

Figure 3.15. Surface expression of EC CD54, CD102 and CD31. EC were harvested using trypsin-EDTA and stained using either isotype control IgG_1 (5 μg/mL, grey line) or mAb against CD54 (A), CD102 (B) or CD31 (C, positive control) (5 μg/mL, black lines) and a FITC-conjugated secondary Ab. EC were washed, fixed in 2% PFA and analysed as in Figure 3.9. Data are representative of 2 independent experiments.

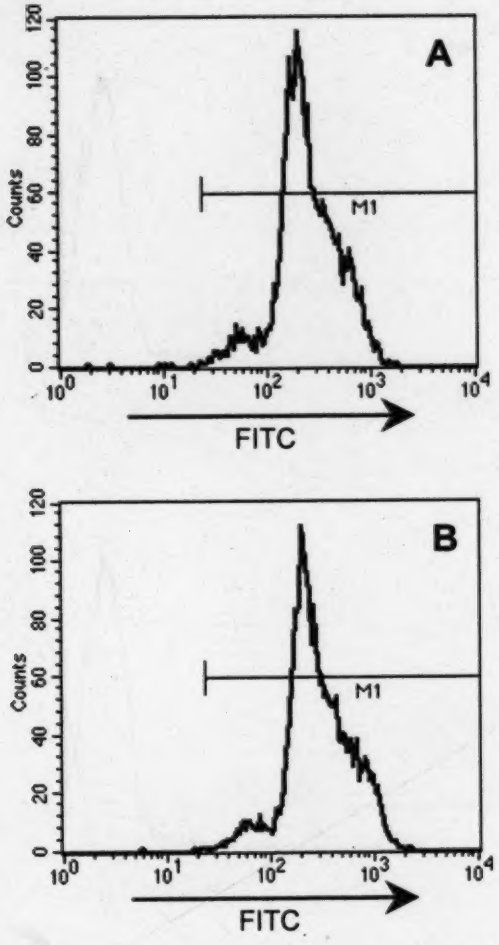

Figure 3.16. Surface expression of lymphocyte CD11a and CD18. Freshly isolated PBL were stained using isotype control IgG_1 (2 μg/mL, grey lines), anti-CD11a mAb (A, 2 μg/mL, black line) or CD18 (B, 1:100 dilution of purified ascites, black line) and a FITC-conjugated secondary Ab. PBL were washed, fixed in 2% PFA and analysed as in Figure 3.9. Data are representative of 2 independent experiments.

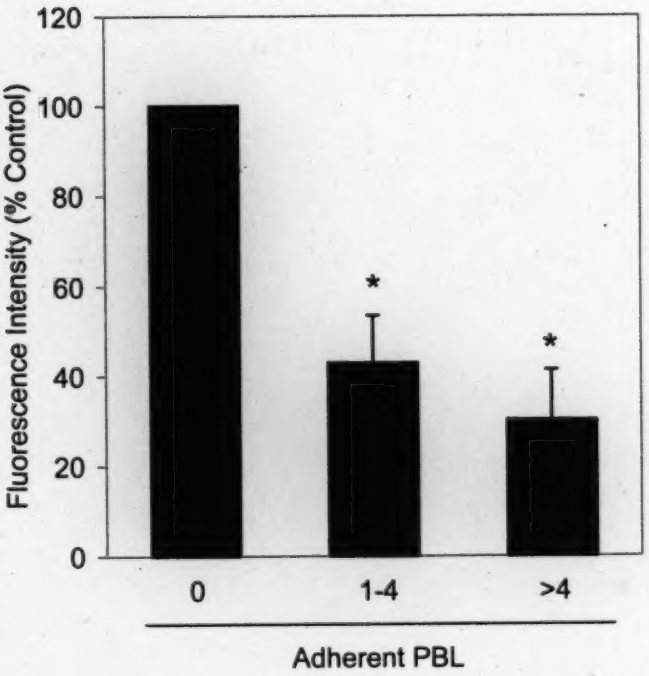

Figure 3.17. Effect of freshly isolated lymphocyte adhesion via CD11a/CD18: CD54/CD102 on endothelial FA fluorescence intensity. PBL were treated with the activating CD11a mAb MEM-83 for 30 minutes and co-cultured with untreated EC for 120 minutes (D). EC were fixed and stained using a FITC:paxillin mAb and an Alexa Fluor 488-conjugated anti-FITC pAb. EC FA paxillin fluorescence was analysed as in Figure 3.6. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group (*$p<0.05$ by ANOVA).

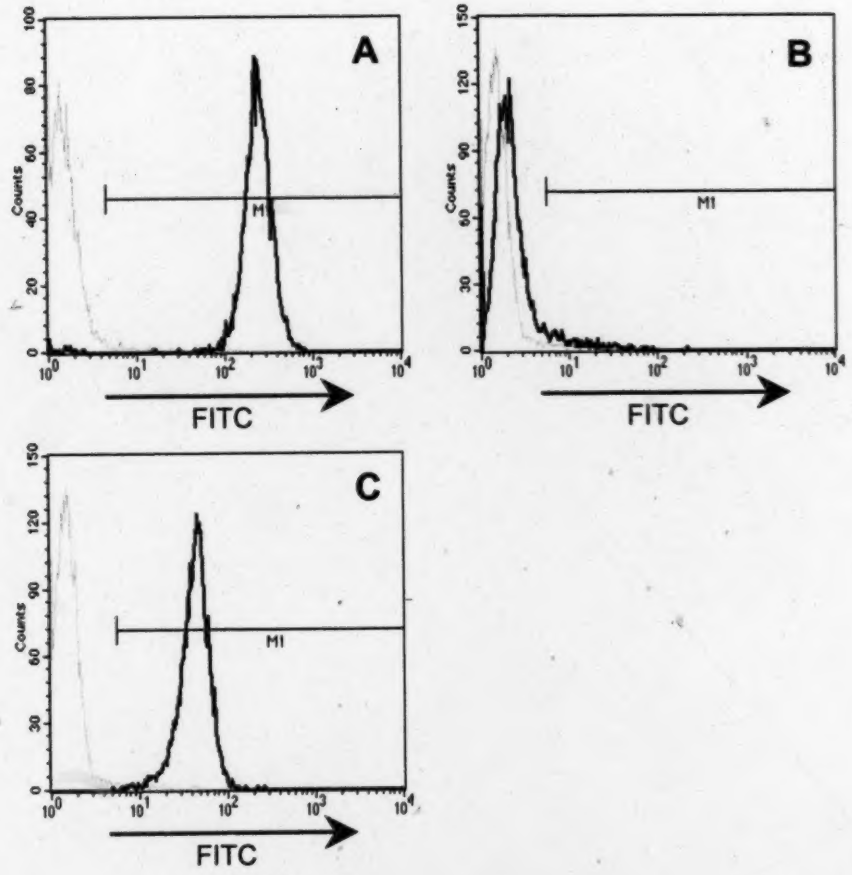

Figure 3.18. EC and JY lymphoblast CD29 surface expression. EC (A) or JY lymphoblasts (B) were stained using isotype control IgG_1 (5 μg/mL, grey line) or anti-CD29 mAb (1:100 dilution of ascites, black line) and a FITC-conjugated secondary Ab. As a positive control, JY cells were stained using anti-CD18 mAb (C, 1:100 dilution of purified ascites). Cells were washed, fixed and analysed as in Figure 3.9. Data are representative of 2 independent experiments.

Figure 3.19. EC and JY lymphoblast CD29 expression by immunoblot. EC (lane 1) or JY lymphoblasts (lane 2) were lysed and immunoblotted for CD29 using mAb TS2/16 (1:1000 dilution of ascites) and biotin-SP-conjugated secondary Ab (0.2 μg/mL). Membrane was exposed to substrate for 5 minutes and exposed to film for 60 seconds. Immunoblot is representative of two independent experiments.

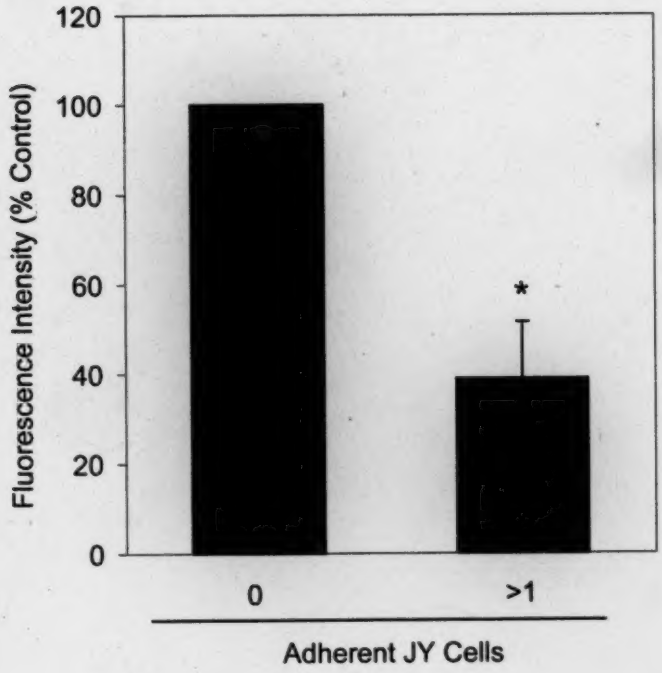

Figure 3.20. Effect of CD29-deficient JY lymphoblast adhesion on endothelial FA fluorescence intensity. JY cells were treated for 30 minutes with CellTracker orange and added to untreated EC. Following 60 minutes of co-culture, EC were fixed, immunostained using for paxillin and analysed as in Figure 3.6. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group.

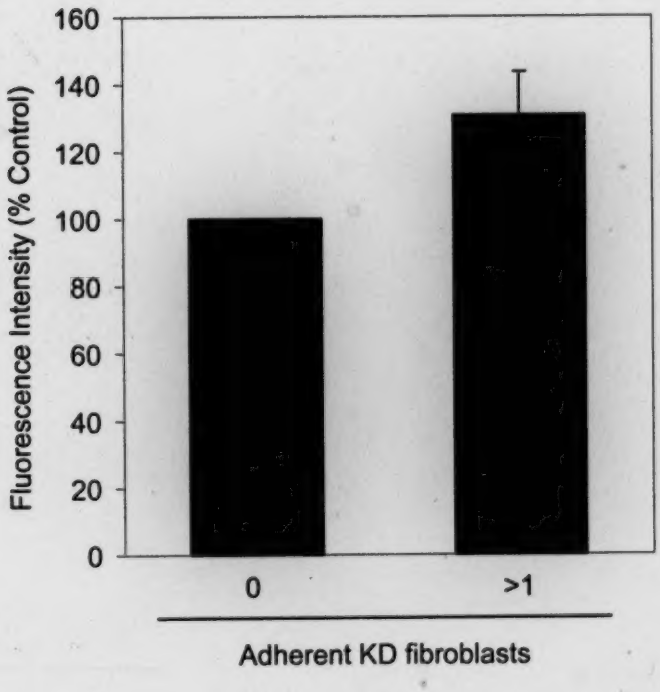

Figure 3.21. Effect of Mer+ KD fibroblast adhesion on endothelial FA fluorescence intensity. KD fibroblasts were treated for 30 minutes with CellTracker orange and added to untreated EC. Following 60 minutes of co-culture, EC were fixed, stained and analysed as in Figure 3.6. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group.

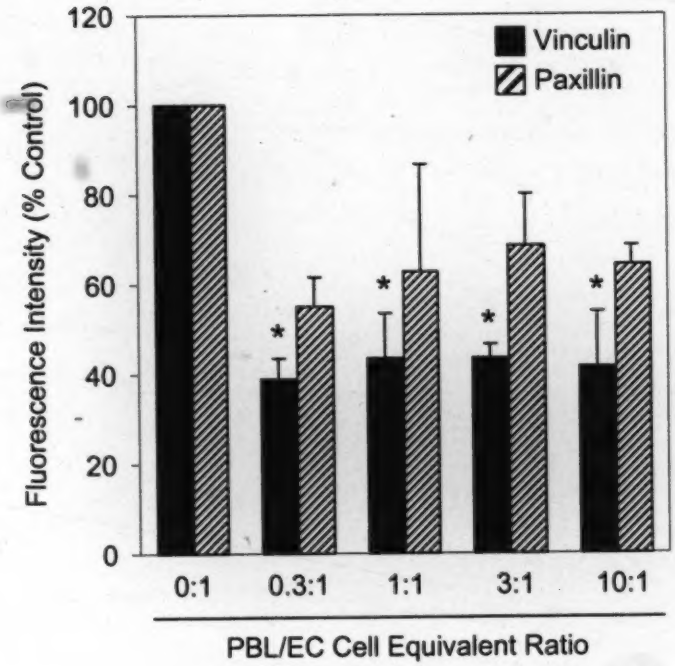

Figure 3.22. Effect of PHA-treated PBL membrane co-culture on endothelial FA fluorescence intensity. PBL were treated for 16-18 hours with 5 μg/mL PHA and 1 nM concanamycin A then lysed in a 5% sucrose buffer with protease inhibitors (Section 2.5). Membranes were added to EC at the indicated cell equivalent ratios. Following 2 hours of co-culture, EC were fixed and stained for FA proteins paxillin and vinculin. Sum fluorescence intensity was calculated for membrane-stimulated EC and normalised to the mean values of sham-treated EC. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group (*$p<0.05$ by ANOVA).

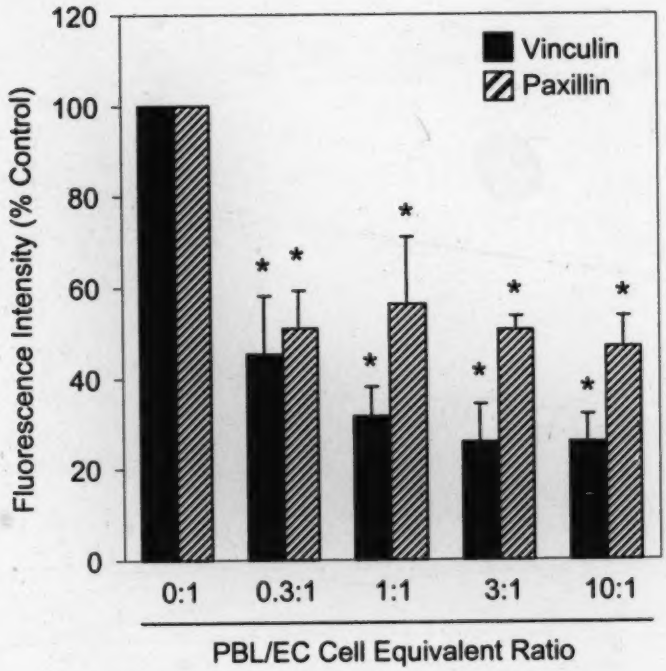

Figure 3.23. Effect of freshly isolated lymphocyte membrane co-culture on endothelial FA fluorescence intensity. PBL were treated for 16-18 hours with 1 nM concanamycin A then lysed as in Figure 3.22. Membranes were added to EC at the indicated cell equivalent ratios. Following 2 hours of co-culture, EC were fixed, stained and analysed as in Figure 3.22. Data represent mean ± SEM of 3 independent experiments, each with 15 EC/group (*$p<0.05$ by ANOVA).

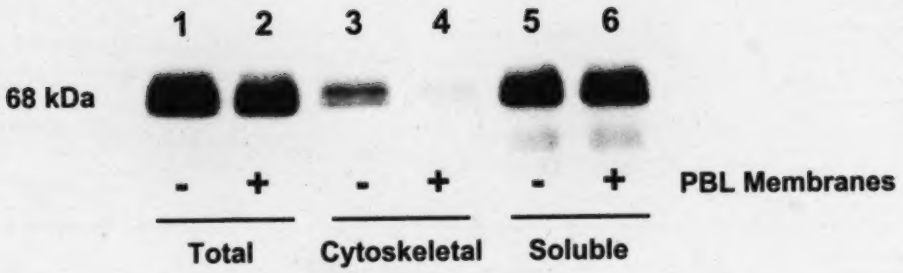

Figure 3.24. Endothelial paxillin levels following stimulation with lymphocyte membranes. EC were unstimulated (lanes 1, 3 and 5) or co-cultured with freshly isolated PBL membranes for 60 minutes (lanes 2, 4 and 6) and assessed by immunoblot. EC were lysed using MLSB (lanes 1 and 2) or MLSB (lanes 3 and 4) following CSK (lanes 5 and 6) and immunoblotted using anti-paxillin mAb and HRP-conjugated secondary Ab. Membrane was exposed to substrate for 2 minutes and exposed to film for 20 seconds. Immunoblot is representative of two independent experiments.

CHAPTER FOUR

General Discussion

The work presented in this thesis demonstrates that stable lymphocyte contact results in the reorganisation of endothelial abluminal matrix receptor adhesion complexes. The time frame of endothelial FA reorganisation correlates with the identified kinetics of PBL transendothelial migration observed under flow conditions *in vitro* [80] and *in vivo* [178]. Endothelial FA remodelling is dependent upon specific lymphocyte adhesion, but not PHA-activation of the PBL. Remodelling cannot be induced by adherent fibroblasts. Finally, attenuation of EC FA fluorescence is independent of PBL CD29.

Genistein treatment of cultured EC tested the validity of the immunocytochemical methods utilised throughout the subsequent studies. Defilippi et al. [175] have shown that FA in genistein- but not sham- or DMSO-treated HUVEC become attenuated. On repetition of these experiments and quantification of the results, I observed endothelial FA remodelling, which was reflected in the measurement as a decrease in fluorescence intensity of FA paxillin.

Leukocyte-dependent reorganisation of EC cytoskeletal structures has been shown to influence leukocyte transendothelial migration. For example, leukocyte adhesion to EC stimulates calcium-dependent myosin light chain phosphorylation and increased endothelial contractility [179;180]. Inhibition of these events

prevents transendothelial migration, as does depolymerisation of endothelial f-actin [181]. Cross-linking of endothelial CD54 can also result in cytoskeletal reorganisation [169;182]. Moreover, structures linked to the cytoskeleton, such as the interendothelial adherens junctions, are remodelled during leukocyte diapedesis [3;89;183]. It has also been shown that endothelial FA remodelling is required for efficient lymphocyte transmigration to a chemokine gradient *in vitro* (R. Moyse and A. Murray, personal communication). When PBL-induced EC FA remodelling is inhibited using an activating CD29 mAb, lymphocyte transendothelial migration is similarly blocked (R. Moyse and A. Murray, personal communication).

PHA-stimulated PBL contact alters the assembly of endothelial matrix adhesion structures, and causes the attenuation of both FA paxillin and vinculin.
EC with adherent PBL develop lamellipodia, and large FA are replaced with small focal contacts. The question is: how are endothelial FA remodelled? To begin to answer this question, one must address the nature of FA dynamics.

Cellular matrix adhesion structures are dynamic protein complexes under constant remodelling [116;150]. Through the use of eGFP-fusion proteins and time-lapse videomicroscopy, FA in stationary fibroblasts *in vitro* can be observed to undergo centripetal migration towards an inner, culling zone. The FA structures remain intact as they migrate, as opposed to randomly dispersing into the cytoplasm. Concomitant with the loss of mature FA is the formation of new,

spiculated focal contacts at the periphery of the cell. These small contacts then develop into larger focal plaques, through integrin aggregation and increased associations with the cytoskeleton and other FA proteins.

PBL adhesion may result in FA attenuation by preventing the formation of large FA from newly formed focal contacts. In the presence of adherent PBL, endothelial focal plaques may continue to undergo centripetal migration while new focal contacts form at the periphery of the cell. However, PBL adhesion may result in the arrest of focal contact maturation, such that large FA cannot form. Alternatively, endothelial FA attenuation may be an active process, occurring by specific protein displacement away from the FA, as opposed to inhibited maturation. However, EC with adherent PBL appear to have an overall increase in the number of focal complexes, which may discredit this model. An alternative to the models involving inhibited maturation or the dissipation of FA proteins into the cytoplasm is FA divergence. That is, following PBL contact, the FA remain structurally intact but separate, with one large focal plaque splitting into several smaller structures. The static images from the immunocytochemistry studies of PBL-induced endothelial FA remodelling do not allow for discrimination between these possibilities.

Based on the work of Zamir et al. [116] and Smilenov et al. [150], I would expect that were EC FA remodelling due to protein displacement or FA divergence, the phenotype of the focal complexes would revert, over time, to that observed in the

absence of PBL contact. The dynamic nature of FA, along with the observation that EC FA attenuation is maintained throughout the period of PBL contact, supports the model of inhibited maturation.

As the immunocytochemical studies did contain the possibility for subjectivity and bias, I attempted a biochemical confirmation of these results. EC co-cultured with PBL membranes exhibited negligible changes in total endothelial paxillin levels. The anti-paxillin mAb used in these studies has previously been shown to recognise a 50 kDa degradation product of the FA protein [184]. In their study, Del Maschio et al. [112] observed degradation of adherens junction catenins following leukocyte adhesion and during their subsequent transendothelial migration. This effect was later shown to have been induced by proteases released during the detergent lysis procedure [113]. In the studies presented here, paxillin was not degraded following PBL membrane stimulation, suggesting that direct proteolysis is not the mechanism by which endothelial FA are remodelled.

Changes in paxillin fluorescence intensity following PBL adhesion may be a result of altered partitioning within the cell following PBL stimulation. To address this question, EC paxillin localisation was studied using differential detergent solubilisation. In support of this model, cytoskeleton-associated paxillin levels were decreased following PBL membrane stimulation, possibly resulting in altered strength of endothelial adhesivity to the extracellular matrix.

The question was posed whether increasing numbers of adherent PHA-treated PBL would enhance endothelial FA attenuation. Lymphocytes have been shown to recruit other PBL to sites of transendothelial migration. Del Pozo et al. [185] have shown that chemokines can induce T lymphocytes to polarise and extend a uropod rich in ICAM molecules into the lumen of the blood vessel. The uropod has been postulated to allow the endothelium-adherent PBL to capture other T cells, resulting in enhanced numbers of transmigrating cells. Similar interactions are also involved in enhancing leukocyte infiltration into sites of inflammation [9;186-188]. Taken together, these studies suggest that leukocytes may migrate co-operatively. Therefore, I expected that PBL would induce FA remodelling in a dose-dependent fashion to facilitate a similar behaviour. However, there was no significant difference in endothelial FA attenuation following adhesion of 1-4 or >4 PHA-treated PBL.

Two models may account for the lack of dose-dependency in PBL adhesion-induced endothelial FA remodelling. First, endothelial FA remodelling may be an "on" or "off" event, without differing degrees. This explanation is supported by the PBL:EC dose-response studies as well as the PBL membrane data. In the presence of cell equivalent ratios of PBL membranes to EC ranging from 0.3:1 to 10:1, there is no significant enhancement of FA attenuation. The second possibility is that the method of analysis used to quantify the fluorescence

intensity within the FA may lack a sufficient degree of sensitivity. The experiments presented here do not distinguish between these possibilities.

Smith and Ford [178] have shown that, following the intravenous injection of radiolabelled PBL, these cells could be detected exiting the high endothelial venules of lymph nodes within 5-10 minutes. Miura et al. [189] studied the migration of subpopulations of fluorophore-labelled PBL into rat Peyer's patches and showed transmigrated PBL entering the interstitium 10-20 minutes after they were seen to adhere. More recently, Cinamon et al. [80] have shown that PBL added to cytokine-activated HUVEC begin transmigrating 5-15 minutes after adhesion to the EC, when subjected to shear stress conditions. In a static adhesion system, I observed similar kinetics for PBL-induced EC FA remodelling. Significant attenuation of EC FA paxillin was present following 15, but not 5, minutes of PBL adhesion. Taken together, these observations suggest a model wherein EC FA remodelling may facilitate PBL transmigration.

Why are endothelial FA not remodelled within five minutes of lymphocyte adhesion? First, a threshold amount of contact time between the PBL and the EC may be required to elicit FA remodelling. This would prevent the remodelling of endothelial FA following transient PBL adhesion, for example during lymphocyte rolling *in vivo*. Alternatively, the endothelial FA may have begun to undergo remodelling, but the resulting phenotype may not be completely established. In the model of PBL-induced inhibition of endothelial FA maturation, FA

disassembly is unaffected, however, subsequent maturation of newly formed focal contacts into larger FA is blocked. Thus, the 5 minute time point may reflect normal FA disassembly, whereas the later time points reflect altered FA maturation. In agreement with this explanation, in Figure 3.5B, small focal plaques can be observed at the outer periphery of the EC following PBL adhesion for 5 minutes.

Endothelial FA remodelling is dependent upon stable lymphocyte adhesion and not a soluble factor or protease elaborated by PBL, as adjacent EC in the PBL/EC co-cultures with no firmly adherent PBL contain well-developed FA. *In vivo* then, microvessel endothelium might remodel matrix adhesions spatially only within those EC with adherent PBL.

Preliminary studies in this laboratory showed no change in endothelial FA fluorescence in the presence of freshly isolated PBL; however, these PBL did not adhere to EC in a sustained manner. When sustained adhesion was induced through β1 and β2 integrin-dependent mechanisms however, PBL-induced endothelial FA remodelling was present. Similar results were present when EC were stimulated with membranes prepared from resting PBL. Thus, prior TcR-mediated PBL activation does not appear to be necessary for the induction of endothelial FA remodelling.

Membranes prepared from PHA-stimulated or freshly isolated PBL promote less paxillin and vinculin aggregation in FA. I have demonstrated that endothelial FA remodelling requires direct, sustained contact and is not induced by a soluble factor. These studies further indicate that the PBL molecule(s) responsible for inducing EC FA remodelling are constitutively present on the membranes. In addition, these studies confirm that resting PBL are able to induce EC FA remodelling following the establishment of adhesion, in the absence of chemokine or activating mAb pre-treatment.

Integrins are expressed by both resting and activated PBL and mediate firm adhesion to the endothelium during diapedesis under flow conditions *in vitro* and extravasation *in vivo*. Further, in the system presented here, the induction of endothelial FA remodelling is dependent upon firm adhesion of the PBL. Taken together, this suggests a model wherein integrin-mediated firm adhesion may be permissive but another molecule signals the induction of EC FA remodelling. This model was further developed from experiments using freshly isolated PBL. The IL-4/MCP-1 approach resulted in EC FA remodelling through CD29-dependent mechanisms. In contrast, the MEM-83 mAb-based approach resulted in CD11a- (and by extension CD18) dependent, CD29-independent EC FA attenuation. Based on these observations, and the availability of a CD29-deficient lymphoid cell line, the role of PBL β1 integrin was addressed.

Endothelial FA remodelling is independent of PBL CD29-based signalling. EC FA remodelling may be integrin-independent, as the effect is observed in the absence of the β1 or β2 integrins in separate experiments using freshly isolated PBL and in the absence of CD29 when using JY lymphoblasts. However, integrins are ideal candidates for the induction of EC FA remodelling, as they are responsible for sustained adhesion during transendothelial migration. Although the phenotypes of integrin-null animals suggest specific and non-redundant roles for these adhesion molecules during PBL trafficking, I would suggest that integrins may function redundantly in this situation, to ensure the induction of EC FA remodelling.

Endothelial FA remodelling is a specific event induced following PBL contact. KD fibroblast cells are unable to elicit the effect, although they are able to adhere in a sustained manner. This suggests specificity of the endothelial response. That is, direct contact does not appear to be the sole requirement for EC FA remodelling, and some PBL-specific molecules may likely be involved in its induction. This idea fits well with an *in vivo* model, as cells undergoing transendothelial migration are predominantly immune-related. Thus, leukocytes may have specialised adhesion molecules which allow them to effect changes in the endothelial cytoskeleton as a means to facilitate transmigration. Other cells, including fibroblasts, may not need to extravasate or induce changes in the endothelium, such as FA remodelling, to facilitate their migration.

An affinity or a signalling change in the EC β1 integrin is required for both PBL-induced endothelial detachment [65] and EC FA remodelling (manuscript submitted). However, the exact mechanism by which the structural changes within the FA occur has yet to be determined. Candidate molecules which may be involved in these events include Src and pp125FAK tyrosine kinases and members of the Rho family of small (20-25 kDa) GTPases.

Activation of pp125FAK and Src-family kinases (SFK) are known to disrupt FA assembly [135], and enhance FA turnover [168]. In agreement with these findings, cells derived from mice lacking FAK and Src protein tyrosine kinases display enhanced FA assembly [134;151]. These phenotypes may result from the inhibitory effect of these kinases on the Rho GTPase family member, RhoA [135;168]. RhoA has been shown to play a role in the formation of large focal plaques, whereas Rac1 regulates the formation of smaller focal contacts and lamellipodia [119], which are observed in EC with adherent PBL. Thus, PBL-mediated induction of endothelial FA remodelling might proceed as follows. Sustained PBL adhesion may induce changes in the activation state of the endothelial β1 integrin, based on the inability of PBL to induce FA remodelling in EC plated in the presence of an activating CD29 mAb (manuscript submitted). Following the initiation of this signal cascade, specific tyrosine kinases (such as Src or FAK) may become activated, resulting in the phosphorylation of various downstream targets. These phosphorylation events may result in the modulation of Rho family member activity, possibly through the inactivation of RhoA and the

activation of Rac1. The signals responsible for endothelial FA remodelling may be further potentiated by the inhibitory effect of Rac1-GTP on RhoA activation. Thus, through such a signalling cascade, although disassembly of endothelial FA remains unaffected, maturation of newly formed FA is abrogated.

Proteases, such as the intracellular thiol protease calpain, can also localise to FA complexes [190] and mediate their disassembly [184;191]. PBL adhesion may be inducing the activation of calpain, resulting in the attenuation of mature EC focal complexes. Huttenlocher et al. [192] have shown that the inhibition of calpain stabilises FA complexes. I would suggest that if PBL-induced EC FA attenuation involves calpain, the protease would be acting upstream of, rather than directly upon, the FA. The lack of total endothelial paxillin loss and the absence of degraded paxillin products concomitant with EC FA attenuation following PBL stimulation supports this model.

The experiments described in this thesis present a PBL-induced endothelial phenomenon (manuscript submitted), which appears to facilitate their diapedesis *in vitro* (R. Moyse and A.Murray, personal communication). Although some requirements for the induction of endothelial FA remodelling have been ascertained, the mechanism and signalling pathway by which these changes occur have yet to be elucidated. Transient transfection of EC with fluorescent fusion proteins in conjunction with real-time videomicroscopy (as in [89;116;150])

would be useful in clarifying the mechanism(s) by which the FA become attenuated.

Further, the definition of the signalling cascade(s) responsible for the induction of this effect will provide a more complete understanding of PBL transmigration. Through selective activation or inhibition of signalling proteins known to localise to, and effect changes in, endothelial matrix receptor complexes, one could assess changes in FA dynamics and the kinetics of PBL trafficking events.

Taken together, these proposed experiments would provide insight into the molecular events surrounding EC cytoskeletal remodelling as physiologic requirements for PBL transmigration in both normal immune surveillance and in robust inflammatory responses. Furthermore, these experiments would serve to assist in the identification of potential targets for therapeutic intervention.

Conclusions

1. Sustained lymphocyte contact results in the remodelling of EC FA.
2. Endothelial FA remodelling occurs between 5 and 15 minutes following sustained lymphocyte adhesion and is compatible with the observed kinetics of lymphocyte transendothelial migration *in vitro* and extravasation *in vivo*.
3. Endothelial FA remodelling is independent of PHA activation based on the ability of stably adherent, freshly isolated (resting) lymphocytes to induce the effect.
4. Membranes prepared from PHA-stimulated and freshly isolated lymphocytes induce endothelial FA remodelling, suggesting that the molecule(s) responsible for inducing this effect are present on the membrane.
5. β1 integrin-deficient JY lymphoblasts induce loss of paxillin from the EC FA, suggesting that CD29:CD106 interactions are not required for the induction of EC FA remodelling.
6. Adherent human fibroblasts are unable to induce paxillin loss from the EC FA, suggesting that the putative signal to induce remodelling is not universally present in human cell membranes, but is found in lymphocytes and a lymphocyte line.
7. Lymphocyte-induced changes in paxillin incorporation into the FA represent redistribution within, and not loss from, the EC.

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